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Oligomerisation of chemokine receptors CXCR1 and CXCR2

A thesis presented for the degree of Doctor of Philosophy

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Division of Biochemistry and Molecular Biology

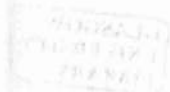
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Abbreviations

BRET	bioluminescence energy transfer
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CFP	cyan fluorescent protein
cGMP	guanosine monophosphate
CNS	central nervous system
DAG	diacylglycerol
DOP	δ opioid receptor
ECL	extracellular loops
ER	endoplasmic reticulum
eYFP	enhanced yellow fluorescent protein
FLIM	fluorescent lifetime imaging
FRET	fluorescent resonance energy transfer
G protein	GTP-binding protein
GABA	γ -amino butyric acid
GAG	glycosaminoglycans
GAP	GTP-ase activating protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GFP	green fluorescent protein
GPCR	G protein coupled receptor
GRK	G protein receptor kinase

GTP	guanosine triphosphate
HEK	human embryonic kidney
ICL	intracellular loops
IL-8	Interleukin-8
IP ₃	inositol (1,4,5) trisphosphate
KOP	κ opioid receptor
LB medium	Luria-Bertani medium
MAPK	mitogen activated protein kinase
MOP	μ opioid receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIP ₂	phosphatidyl inositol (4,5) triphosphate
PKA	protein kinase A
PKC	protein kinase C
PMNs	polymorphonuclear leukocytes
RGS	regulator of G protein signalling
Rluc	<i>Renilla</i> luciferase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T.A.E.	tris acetate EDTA
T.E.	tris EDTA
TM	transmembrane domains
Tr-FRET	Time resolved FRET

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Abstract

Chemokine receptors CXCR1 and CXCR2 are expressed on several immune cell types including granulocytes and monocytes. The two receptors share 78% sequence identity and

primarily function to mediate chemotaxis. Homo- and hetero-oligomerisation of CXCR1 and CXCR2 was investigated in this study.

CXCR1 and CXCR2 were modified with either N- or C-terminal epitope tags in order to facilitate the detection of receptor oligomers. The modified receptors were predominantly cell surface expressed and underwent prominent internalisation following exposure to Interleukin-8 (IL-8). The modifications were found to have no effect on affinity for IL-8 determined by competition radioligand binding assays. The modified receptors retained the ability to couple to G proteins assessed by agonist-induced reduction in forskolin-stimulated cAMP levels. Co-immunoprecipitation experiments demonstrated the existence of constitutive CXCR1 and CXCR2 homo-oligomers and CXCR1/CXCR2 hetero-oligomers following expression in HEK293T cells. Single cell FRET imaging experiments also confirmed constitutive CXCR1 and CXCR2 homo- and hetero-oligomerisation. A lower level of energy transfer was observed between both CXCR1 and CXCR2 and the less homologous α_{1A} -adrenoceptor. Saturation BRET experiments indicated that CXCR1/CXCR2 homo- and hetero-oligomers formed with equal propensity. This approach also indicated that the interaction between CXCR1 and the α_{1A} -adrenoceptor detected in FRET studies was non-specific as a linear relationship between donor and acceptor was shown to exist. Tr-FRET experiments indicated that constitutive CXCR1 and CXCR2 homo- and hetero-oligomers exist pre-formed at the cell surface. The effect of agonist on oligomerisation was investigated. Experiments using BRET and Tr-FRET did not demonstrate any modulation of energy transfer following exposure to IL-8.

A novel endoplasmic reticulum (ER) trapping strategy was developed in order to investigate the location and selectivity of oligomer formation. CXCR1 was modified by the

addition of an ER retention sequence to the C terminal tail that prevented expression of the receptor at the cell surface. Upon co-expression with wild type CXCR1 and CXCR2, CXCR1-ER reduced their cell surface expression. This suggested that oligomerisation between the wild type receptors and CXCR1-ER was initiated during protein synthesis and maturation and prior to cell surface delivery. Cell surface expression of the α_{1A} -adrenoceptor was unaffected by co-expression of CXCR1-ER indicating a lack of interaction. Despite cell surface constitutive CXCR1/CXCR2 hetero-oligomers being indicated by Tr-FRET, no co-internalisation of CXCR1 and CXCR2 was observed in response to GRO- α , a ligand specific for CXCR2.

Expression of opioid receptors on immune cells has been demonstrated and opioid agonists have been demonstrated to modulate chemotaxis. CXCR2 is also expressed in the brain. Co-immunoprecipitation experiments indicated oligomerisation between CXCR2 and DOP, MOP and KOP receptors. Single cell FRET also indicated oligomerisation between CXCR2 and the opioid receptors. This highlights the potential existence of chemokine and opioid hetero-oligomers in physiologically relevant settings that could be of great potential interest in drug development for the treatment of inflammatory conditions.

1.0 Introduction

1.1 G protein coupled receptors (GPCRs)

GPCRs are a superfamily of receptors that play an important role in signal transduction. Analysis of GPCR sequences reveals that >800 GPCRs are encoded in the human genome (Fredriksson *et al.*, 2003). The majority of GPCRs identified are orphan receptors for which no endogenous ligand has been found and the receptor function has not been defined (Karnik *et al.*, 2003). GPCR dysfunction has been shown to be associated with human disease and many GPCRs are targets for pharmaceuticals and drugs of abuse. A report from 2002 indicated that >50% of all drugs available in the clinic target GPCRs and 20% of those drugs present in the top 50 prescribed drugs worldwide target GPCRs (Ma and Zimmel, 2002). Members of the GPCR superfamily can represent up to 30% of the portfolio of many pharmaceutical companies, demonstrating the importance of these receptors as drug targets (Klabunde and Hessler, 2002).

GPCRs function to transduce a signal received via an extracellular mechanism into the cell. The majority of this intracellular signalling involves the activation of hetero-trimeric G proteins that serve to trigger multiple signal transduction pathways within the cell.

1.1.1 Structure of GPCRs

Despite low sequence homology observed across the GPCR superfamily, a similar basic structure has been identified. Recently the crystal structure of the GPCR bovine rhodopsin at 2.8Å was reported revealing a highly organised heptahelical arrangement of

transmembrane (TM) domains (Palczewski *et al.*, 2000). This was in agreement with data generated from earlier studies utilising electron microscopy (Unger *et al.*, 1997). Rhodopsin was shown to consist of a central core domain of seven TM helices connected via three extracellular loops (ECLs) and three intracellular loops (ICLs).

The N-terminal region of GPCRs varies considerably in length between individual receptors and can be important in ligand binding. All GPCRs possess one or more N-linked glycosylation sites (Asn-X-Ser/Thr) where X is any amino acid except proline or asparagine (Kristiansen, 2004). This region has been implicated in determining the correct trafficking of the receptor to the plasma membrane (Petaja-Repo *et al.*, 2000). For several GPCRs prevention of receptor N-terminal glycosylation resulted in reduced cell surface expression but had no effect on ligand binding or function of those receptors that were plasma membrane expressed (George *et al.*, 1986; Ji *et al.*, 1990; Liu *et al.*, 1993; Davidson *et al.*, 1995; Ray *et al.*, 1998). Some GPCRs also possess cysteine residues within this region thought to be important in correct protein folding (Kristiansen, 2004).

The seven transmembrane domains vary in length and form α helices. The crystal structure of bovine rhodopsin has revealed that the transmembrane domains are kinked or tilted. This is due to the presence of certain amino acids capable of distorting the helical domains. The helices are arranged in a counter-clockwise order. The helices of TM I and II in bovine rhodopsin were found to be tilted from the plane of the membrane by 25° while TM III demonstrated a 33° tilt. The C-terminal end of TM III contains a highly conserved DRY (Asp-Arg-Tyr) motif, commonly identified in rhodopsin-like receptors, that has been implicated in the regulation of receptor interaction with its G protein (Franke *et al.*, 1990). TM IV and VI were found to be almost perpendicular to the membrane while TM V tilts at

26° (Teller *et al.*, 2001). TM VII possesses a highly conserved NPXXXY motif. This motif is thought to be involved in forming a structural domain that facilitates an interaction with TM VI, important in conferring the conformation relating to the inactive state. The crystal structure also highlighted the presence of an amphipathic cytoplasmic helix termed helix VIII located at the extension of TM VII. This helix is tilted at 90° to the membrane and plays a role in G protein coupling in rhodopsin (Teller *et al.*, 2001).

The crystal structure of rhodopsin yielded little data concerning the cytoplasmic regions of the receptors. However, these regions have been demonstrated to play an important role in G protein coupling with the second and third intracellular loop and C-terminal tail all implicated (Wess *et al.*, 1998; Cotecchia *et al.*, 1992; O'Dowd *et al.*, 1988). The third intracellular loop of GPCRs also demonstrate several serine and threonine residues that provide sites for receptor regulation. Phosphorylation of these sites by receptor kinases can trigger receptor desensitisation and internalisation.

The extracellular loops of rhodopsin possess two highly conserved cysteine residues in ECL II and the N-terminal end of TM III (Cys¹¹⁰ and Cys¹⁸⁷ respectively) that form a disulphide bond (Teller *et al.*, 2001). This interaction has been demonstrated to provide stabilisation of receptor structure (Dohlman *et al.*, 1990). Site-directed mutagenesis of cysteine residues (Cys¹⁰⁶ and Cys¹⁸⁴) within the β_2 -adrenoceptor demonstrated a reduced ability to bind ligand and destabilisation of receptor tertiary structure (Dixon *et al.*, 1988; Dohlman *et al.*, 1990).

A further cysteine residue is located in the membrane proximal C-terminal region of several GPCRs that has been identified as a potential site for palmitoylation (O'Dowd *et*

et al., 1989). Palmitoylation is a post-translational modification in which the fatty carbon chain palmitic acid is attached to a cysteine residue via a thioester bond. This modification serves as an anchor into the membrane, creating a fourth intracellular loop termed helix VIII, discussed previously. This helix has been shown to affect the coupling of the receptor to the G protein (Ganter *et al.*, 1992; Milligan *et al.*, 1995a). The C-terminal tail also possesses several sites that enable receptor phosphorylation by receptor kinases, inducing receptor desensitisation (Carman *et al.*, 1998).

1.1.2 GPCR family classification

GPCRs are capable of transducing signals initiated by ligand binding to the receptor. A diverse range of ligands can activate GPCRs including biogenic amines, amino acids, lipids and peptides. Ligand binding can trigger heterotrimeric G protein dependent and independent signalling cascades as shown in Figure 1.1. The GPCR superfamily is subdivided into five subclasses based on both sequence similarity and pharmacological nature of the ligand. Family 1 or rhodopsin-like receptors represent the largest class in the GPCR superfamily. Members of this family have short N-terminal domains and possess several highly conserved amino acid residues within each transmembrane domain. This class can be further broken down into three subgroups based upon the ligand-binding site. Class 1a includes the rhodopsin and adrenergic receptors for which the ligand-binding site is located within the seven transmembrane domains. The second subclass, family 1b, includes receptors for which the binding site includes the N-terminal, extracellular loops and parts of the TM domains. This includes peptide receptors such as the chemokine, opioid and somatostatin receptors. Family 1c incorporates receptors that bind their ligands via a large extracellular domain although some contact between the ligands and ECL 1 and

3 also occurs. Members of this family include receptors for glycoprotein hormones such as luteinising hormone.

Family 2 or secretin-like receptors are a smaller class of receptors. Members of this family commonly possess a larger N-terminal than observed for family 1 GPCRs and contains several cysteine residues that form a network of disulphide bridges. This subtype includes receptors for ligands such as secretin and vasoactive intestinal polypeptide (Davletov *et al.*, 1998).

Family 3 or metabotropic-glutamate-receptor-like receptors includes the calcium sensing and GABAB receptors (Kaupmann *et al.*, 1997; Pin and Bockaert, 1995). This family also contains putative pheromone receptors termed VRs and G₀-VN (Bargmann, 1997). This family is characterised by a long amino terminus, often between 500-600 residues, which folds producing a separate ligand-binding domain. The crystal structure of the metabotropic glutamate receptor mglu₁ has been resolved and this demonstrated that the ligand-binding domain, present in the amino terminal, forms a disulphide-linked dimer (Kunishima *et al.*, 2000). This structure is thought to open and close in response to agonist binding and has been likened to the mechanism of the Venus flytrap.

The fourth class of receptors include the pheromone receptors (VNs) that bind to the G_i class of G protein (Dulac and Axel, 1995). Family 5 includes the frizzled/smoothed receptors involved in embryonic development. In mammals there are 10 frizzled and 1 smoothed receptors that are structurally similar to family 2 GPCRs (Foord *et al.*, 2002).

Variations on the structure and conserved residues of GPCRs can be observed throughout the classification system illustrated in Figure 1.2. The majority of family 1 GPCRs are thought to possess a structure homologous to that elucidated for rhodopsin. For family 2 a similar overall morphology to family 1 receptor structure has been hypothesised, however, the palmitoylation site is missing and conserved residues and motifs vary from family 1. The structural organisation of the TM regions has not been determined but are thought to vary significantly from that observed for family 1 receptors due to the variation in amino acid sequence, particularly concerning the conserved prolines that confer the tilting of the TM domains identified in rhodopsin. Family 3 are extremely distinct from family 1 GPCRs sharing only two conserved cysteines in extracellular loop 2 and TM domain III, forming a putative disulphide bridge. The third intracellular loop of this family is short and highly conserved. As with the family 2 GPCRs, little can be determined at this time regarding the orientation of the TM domains (George *et al.*, 2002).

1.1.3 Ligand induced activation of GPCRs

The molecular basis of GPCR activation has been best studied for rhodopsin. Rhodopsin is involved in the detection of light energy and upon binding of a photon transduces a neuronal signal directed to the secondary neurons of the retina. In rhodopsin the inverse agonist, 11-*cis*-retinal, is covalently bound to the receptor (Teller *et al.*, 2001; Meng *et al.*, 2001). Generally, binding of inverse agonists can initiate the transition of a GPCR from the native partially active state to an inactive state (Gether *et al.*, 2000). This is also the case for rhodopsin, which exists in two forms. The rhodopsin form is the inactive form of the receptor while the opsin form exists in a partially active state (Meng *et al.*, 2001). Upon

photon absorption the 11-*cis*-retinal ligand isomerises to all-*trans*-retinal and induces several conformational changes in the receptor (Teller *et al.*, 2001; Subramaniam *et al.*, 1999; Meng *et al.*, 2001). This is an extremely rapid process completed in less than 200fs (Schoellein *et al.*, 1991). These conformational changes trigger disruption of a salt bridge formed between TM III and TM VII. Analogous mechanisms of activation have been demonstrated for amine receptors (Porter *et al.*, 1996) and also peptide receptors however in this family salt bridge disruption was uncommon although residue displacement of TM III was observed (Miura *et al.*, 1999; Ji *et al.*, 1998).

It is evident that the ligand induced conformational changes in GPCRs are essential for correct function (Kenakin, 2002). Studies have been performed investigating the homology of the rhodopsin mechanism of activation throughout the GPCR family. These reports have demonstrated that separation of TM III and VI is a common mechanism in GPCR activation (Sheikh *et al.*, 1996; Miura *et al.*, 2003; Gether *et al.*, 1997; Ballesteros *et al.*, 2001; Sheikh *et al.*, 1999; Shapiro *et al.*, 2002). Studies in which the movement of TM III and VI was hindered artificially prevented G protein activation (Meng *et al.*, 2001). The degree of movement of TM VI was greater than that observed for TM III and TM VII, resulting in a structural change in which the inner faces of TM II, III, VI and VII become more exposed and the cytoplasmic ends of TM IV and V become less exposed, facilitating G protein activation. The movement of the TM helices also acts to initiate changes in the cytoplasmic domains, ensuring G protein binding and activation.

1.1.4 G proteins

Upon ligand activation, GPCRs associate with guanine nucleotide binding proteins (G proteins). G proteins act as mediators of the receptor-activation signal and are heterotrimeric consisting of α , β and γ subunits. In the inactive state GDP is bound to the α subunit which associates with β and γ subunits, forming an inactive heterotrimer. Upon activation by ligand binding, the receptor undergoes a conformational change resulting in increased affinity for G protein. This permits the exchange of GDP for GTP, which reduces the affinity of the interaction between α and $\beta\gamma$ complex and the heterotrimer dissociates. Both α and the $\beta\gamma$ subunits can then interact with several effectors transducing the signal. The activated state lasts until GTP is hydrolysed to GDP by the intrinsic GTPase activity of the $G\alpha$ subunits. The rate of this hydrolysis is under the regulation of GTP-ase activating proteins (GAPs) termed regulators of G protein signalling (RGS proteins) (Siderovski *et al.*, 1996; Koelle and Horvitz, 1996). Each RGS protein demonstrates a 120 amino acid sequence termed the RGS domain. This region forms a nine alpha helix bundle that contacts the $G\alpha$ switch regions (Tesmer *et al.*, 1997). Many RGS proteins stimulate rapid GTP hydrolysis and attenuate GPCR signalling (Neubig and Siderovski, 2002). This intervention can be at the level of the receptor via interaction with PDZ domains or the G protein level.

1.1.5 G protein α subunit

The heterotrimeric G proteins are composed of α subunits (39-45kDa), β subunits (35-39 kDa) and γ subunits (6-8kDa). At least 28 distinct α subunits have been identified that are the products of 16 different genes. G proteins are classified into four subfamilies with

respect to the $G\alpha$ amino acid sequence similarity; G_i , G_s , G_q and G_{12} . The $G\alpha_i$ family includes $G\alpha_{i1-3}$ which act to inhibit adenylyl cyclase (Cabrera-vera *et al.*, 2003). $G\alpha_{i1}$ is widely expressed, preferentially in neuronal systems, however $G\alpha_{i3}$ is preferentially expressed in non-neuronal systems. $G\alpha_{i2}$ is ubiquitously expressed (Ellis *et al.*, 2004). $G\alpha_{tr-c}$ stimulates cGMP phosphodiesterases (Mochizuki *et al.*, 1999; Pierce *et al.*, 2002) and are expressed in retinal rods and cones respectively. $G\alpha_{o1-2}$ are also members of this family and are expressed in neuronal and neuroendocrine cells and have been shown to inhibit adenylyl cyclase and voltage-dependent calcium channels and stimulate GIRK and phospholipase C (Hsu *et al.*, 1990). $G\alpha_{gust}$ is a further member of this family expressed in taste and neuronal cells while $G\alpha_z$ is found in endocrine cells and platelets. $G\alpha_{gust}$ stimulates cGMP phosphodiesterase while $G\alpha_z$ inhibits adenylyl cyclase (Taussig and Gilman, 1995).

The G_q family includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$ (Pierce *et al.*, 2002; Cabrera-vera *et al.*, 2003; Willets *et al.*, 2003) and all act to stimulate phospholipase activity. G_q family members are broadly expressed with $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$ expressed in haematopoietic cells and tissues and $G\alpha_q$ ubiquitously expressed. $G\alpha_{11}$ is also broadly expressed but is not found in haematopoietic cells. G_s family members include several long and short splice variants. They act to stimulate adenylyl cyclase activity and are widely expressed (Pierce *et al.*, 2002). G_{12} consists of two family members termed $G\alpha_{12}$ and $G\alpha_{13}$ that are ubiquitously expressed. This family has been implicated in the regulation of Ras homology (Rho) guanine nucleotide exchange factors (RhoGEFs) and GTP-ase activating protein of Ras. This can lead to stimulation of Rho and stress fiber formation and activation of Ras (Jiang *et al.*, 1998; Pierce *et al.*, 2002).

G α subunits are essentially enzymes that possess inherent GTPase activity and are composed of two domains. The first domain is responsible for binding and hydrolyzing GTP to GDP while the second confers a unique helical domain that buries the GTP in the core of the protein (Noel *et al.*, 1993; Coleman *et al.*, 1994). Structurally it is composed of 5 α helices surrounding a 6-stranded β sheet, binding the phosphate and the guanine moiety of GTP (Sprang, 1997). Most G α proteins can act as a substrate for mono-ADP ribosylation which is catalysed by either cholera toxin for G α_s and G α_t or pertussis toxin for G α_i and G α_o and G α_q . Pertussis toxin catalyses the covalent binding of ADP ribose to a cysteine residue that is located four amino acids from the C-terminal tail. This results in the uncoupling of the G α subunit from the receptor as the activation of the subunit is inhibited. Cholera toxin specifically targets an arginine residue in the G α subunit that inhibits the inherent GTPase function resulting in constitutive activation of these subunits (Hepler and Gilman, 1992).

The G α subunits are subject to several modifications. G α_i undergoes N-myristoylation that occurs following removal of the initiating methionine residue (Gordon *et al.*, 2001). All other G proteins except G α_t are subject to palmitoylation. These modifications are thought to permit the membrane attachment of the α subunits. This has been demonstrated as palmitoylation deficient mutants of G α_s demonstrate a reduced capacity to associate with the membrane (Wedegaertner *et al.*, 1994). These N-terminal modifications also increase the affinity for $\beta\gamma$ subunits (Linder *et al.*, 1991; Milligan *et al.*, 1995b). The C-terminal region of G α contains a region thought to be involved in the specificity of receptor G-protein interaction (Hamm *et al.*, 1996).

1.1.6 G protein $\beta\gamma$ subunits

Five different $G\beta$ and twelve different $G\gamma$ subunits have been identified. The expression pattern of the β subunits varies considerably. $G\beta_1$ and $G\beta_2$ are ubiquitously expressed while $G\beta_3$ is expressed in cone cells and taste cells. $G\beta_4$ is found in the brain, lung and placenta while $G\beta_5$ is found in neuronal, lung, lymph and ovarian cells (Ellis *et al.*, 2004). The γ subunits are widely expressed throughout the body. $G\gamma_1$ is expressed in retinal rods and neuronal cells while $G\gamma_{2-7}$ and $G\gamma_{10-13}$ have been identified throughout the body especially in neuronal cells. $G\gamma_9$ is expressed in retinal cones. The large number of β and γ subunits give rise to numerous possible combinations. Most combinations are functional however some are unable to form complexes, for example $G\beta_2$ is unable to form a functional dimer with $G\gamma_1$ (Ellis *et al.*, 2004).

The structure of the $G\beta$ subunit is a 7-membered propeller structure based on its seven WD-40 repeats (Sondek *et al.*, 1996; Lambright *et al.*, 1994; Wall *et al.*, 1995). The γ subunit interacts with $G\beta$ via an N-terminal coiled-coil interaction and also at numerous contact points along the base of β , forming a strong functional complex that can be considered as one unit. This association is so strong that dissociation can only be achieved by protein denaturation. The γ subunits are either farnesylated or geranylgeranylated and this serves to anchor the unit to the membrane (Wedegaertner *et al.*, 1995).

The interaction of $G\beta\gamma$ subunit with $G\alpha$ occurs via an interaction of the N-terminal helical domain of $G\alpha$ with the propeller structure of the β subunit (Lambright *et al.*, 1996). As

mentioned previously, G protein activation by ligand binding to the GPCR triggers the exchange of GDP for GTP on $G\alpha$, causing a conformational change within $G\alpha$ leading to reduced affinity for the $\beta\gamma$ subunits and the heterotrimer to dissociate into $G\alpha$ and $G\beta\gamma$ subunits.

Several effectors of $G\beta\gamma$ exist. The $G\beta\gamma$ subunits act to inhibit adenylyl cyclase I while stimulating the function of adenylyl cyclase II, IV and VII. They also act to stimulate G-protein regulated inward rectifier K^+ (GIRK) channels, phospholipase $C\beta$ isoforms (PLC β), and G-protein coupled receptor kinases (GRK) 2 and 3 (Ellis *et al.*, 2004).

1.1.6 GPCR desensitisation and internalisation

As mentioned previously, ligand binding to GPCRs can trigger the activation or attenuation of several signalling pathways. However, following stimulus GPCRs become desensitised and lose cellular sensitivity (Ferguson, 2001; Claing *et al.*, 2002). Short-term desensitisation is mediated, in part, by the phosphorylation of residues within the C-terminal tail by G protein receptor kinases (GRKs). Long-term loss of cellular sensitivity can involve the down regulation of receptors by protein degradation and decreased receptor synthesis. The C-terminal tail of GPCRs contains several serine and threonine residues that can determine the intracellular trafficking and fate by providing phosphorylation sites for several protein kinases (Smith and Scott, 2002; Tobin, 2002).

Desensitisation can be either heterologous or homologous. Heterologous desensitisation is a process that does not require agonist activation of the target GPCR. In this situation the activation of a distinct GPCR can result in the activation of intracellular kinases that can

phosphorylate other non-activated GPCRs, specifically at sites involved in G protein coupling interfering with G protein function. There are several kinases involved including protein kinase A (PKA) and protein kinase C (PKC) (Ferguson, 2001). Homologous desensitisation is a process for which agonist occupation is required for desensitisation of that GPCR (Claing *et al.*, 2002).

The binding of ligand to the GPCR triggers phosphorylation by GRKs. There are currently seven GRKs identified that share considerable sequence homology. The structure of the GRK family members consists of a central catalytic domain, an amino terminal domain important in substrate recognition and a carboxyl terminal domain that contributes to the plasma membrane targeting of the kinase (Ferguson, 2001). GRKs also possess an RGS-like domain in the amino terminal which suggests that they may play a role in regulating the G protein as well as regulating signalling at the level of the receptor (Carman *et al.*, 1999; Sallse *et al.*, 2000). GRK1 and GRK7 are primarily involved in the regulation of rhodopsin (Shichi and Somers, 1978; Weiss *et al.*, 1998). GRK2 and 3 were initially identified as β ARK1 and β ARK2 responsible for the phosphorylation of the β adrenoceptor but have subsequently been shown to have widespread expression (Benovic *et al.*, 1986; Benovic *et al.*, 1991). GRK4-6 are widely expressed in many cell types (Premont *et al.*, 1994; Sallse *et al.*, 1994; Kunapuli and Benovic, 1993; Benovic and Gomez, 1993). Several serine and threonine residues are located within the C-terminal tail of many GPCRs, providing phosphorylation sites for the GRKs. However, GRKs have also been shown to phosphorylate residues within intracellular loop 3 for the human M2 muscarinic and α_{2A} adrenoceptors (Ferguson, 2001). Although no consensus site for GRK activity has been determined, the presence of acidic amino acids proximal to the phosphorylation site favours GRK2 mediated phosphorylation (Onorato *et al.*, 1991; Chen

et al., 1993). GRKs preferentially phosphorylate receptors that are in the agonist-occupied conformation (Luttrell and Lefkowitz, 2002).

Phosphorylation of receptors by GRKs alone has little effect on receptor-G protein coupling but instead serves to increase the affinity of the receptor for cytosolic cofactors termed arrestins. Arrestin binding sterically hinders G protein coupling, serving to uncouple the receptor from the G protein and target the receptor for endocytosis (Benovic *et al.*, 1987; Lohse *et al.*, 1990; Pippig *et al.*, 1993). To date four members of the arrestin family have been identified. These are visual arrestin and cone arrestin that are localised primarily in the retina (Shinohara *et al.*, 1987; Yamaki *et al.*, 1987). Two β -arrestins have been isolated; β -arrestin1 and β -arrestin2 (Lohse *et al.*, 1990; Attramadal *et al.*, 1992). β -arrestins are ubiquitously expressed but are predominantly located in neuronal tissues and the spleen (Attramadal *et al.*, 1992). All members of the arrestin family are capable of binding to ligand-activated GPCRs that have been phosphorylated by GRKs.

β -arrestins serve as endocytic adaptor proteins which can target GPCRs for internalisation via clathrin-coated vesicles (Ferguson *et al.*, 1996; Zhang *et al.*, 1996). GPCR internalisation is an important process in signal attenuation, receptor resensitisation and down-regulation. β -arrestins are capable of binding to the β 2-adaptin subunit of the heterotetrameric AP-2 adaptor complex (Goodman *et al.*, 1997; Laporte *et al.*, 1999). The AP-2 adaptor complex then targets the receptors to the clathrin endocytic pathway by binding clathrin and dynamin and initiates clathrin pit formation (Kirchhausen, 1999). Dynamin is a large GTP-ase molecule that is required for the budding of clathrin-coated pits from the receptor (Zhang *et al.*, 1996).

Receptor endocytosis is followed by the targeting of the receptor to either recycling pathways or to lysosomes for degradation (Kristiansen *et al.*, 2004). Receptor resensitisation involves receptor dephosphorylation and dissociation from its ligand.

The time frame over which these events occur is seconds for GPCR phosphorylation, minutes for receptor internalisation and hours for receptor downregulation.

Recently β -arrestins have been implicated as being GPCR signal transducers. β -arrestins have been demonstrated to interact with a number of other signalling proteins including c-Src (Luttrell *et al.*, 1999), Hck (Barlic *et al.*, 2000), c-Fgr (Barlic *et al.*, 2000), MAPKs and Raf (DeFea *et al.*, 2000). The formation of a complex between the GPCR and β -arrestin can act as a scaffold permitting the interaction of these signalling molecules, thereby allowing β -arrestin to terminate receptor-G protein coupling while initiating alternative signal transduction cascades potentially important in the regulation of cellular processes.

1.2 GPCR Oligomerisation

Traditional models of GPCR ligand binding and signal transduction have always portrayed GPCRs as monomeric signalling units. However, oligomerisation had been suggested from data generated over twenty years ago. Complex radioligand binding techniques using both agonists and antagonists generated data suggesting positive or negative cooperativity between ligands. This result was thought to be due to interactions between receptor monomers (Hazum *et al.*, 1982; Limbird and Leftkowitz, 1976; Limbird *et al.*, 1975; Mattera *et al.*, 1985; Potter *et al.*, 1988). Indirect evidence of GPCR oligomerisation was provided by experiments performed on the gonadotropin releasing hormone receptor (GnRH). This study demonstrated that a bivalent antibody directed at a GnRH receptor

antagonist could trigger receptor aggregation and signalling (Conn *et al.*, 1982). Functional evidence of GPCR oligomerisation was suggested from radiation inactivation experiments that demonstrated that GPCRs function as complexes larger than predicted by their monomeric structure (Conn *et al.*, 1985; Lilly *et al.*, 1983; Venter *et al.*, 1983). Receptor affinity labelling studies also identified higher molecular weight species that could correspond to receptor oligomers (Herberg *et al.*, 1984; Avissar *et al.*, 1983).

The initial stimulus fuelling investigation into GPCR oligomerisation was the discovery that the GABA_B receptor was in fact an obligate heterodimer of two distinct subunits, GABA_BR1 and GABA_BR2 (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998; Sullivan *et al.*, 2000). The GABA_BR1 subunit was initially identified and although it possessed a seven transmembrane structure, it could not account for the pharmacology observed for the native GABA_B receptor. The GABA_BR1 subunit was unable to traffic to the cell surface when expressed in heterologous systems. Subsequent identification of the GABA_BR2 subunit revealed the heterodimer pair involved in conferring the observed pharmacology of the GABA_B receptor. An endoplasmic reticulum (ER) retention motif was identified within the C-terminal tail of the GABA_BR1. Interaction with the C-terminal tail of the GABA_BR2 subunit masks the retention signal, permitting export from the ER and trafficking of the heterodimer complex to the cell surface (Margeta-Mitrovic *et al.*, 2000; Pagano *et al.*, 2001).

1.2.1 Techniques for detection of oligomerisation

1.2.1.1 Functional reconstitution

Functional reconstitution experiments have been used to demonstrate GPCR oligomerisation. This technique utilises non-functional mutants or chimeric receptors that are unable to signal when singly expressed, but upon co-expression with a receptor possessing a different mutation or chimera functionality is restored. A study performed by Monnot *et al.* (1996) investigating oligomerisation of the angiotensin AT₁ receptor used this approach. Two point mutant forms of the AT₁ receptor were generated that were incapable of binding ligand when expressed alone. However, when co-expressed ligand binding was restored. The trans-complementation observed upon co-expression of the mutant constructs is strongly suggestive of protein: protein interactions occurring.

1.2.1.2 Co-immunoprecipitation

Co-immunoprecipitation is a biochemical technique that utilises two differentially tagged receptor forms and immunoprecipitation and subsequent protein detection with antibodies directed against the epitope tags. In 1996 Hebert *et al.*, utilised differentially tagged β_2 -adrenoceptors in this approach to detect homo-oligomerisation. Using c-Myc and HHA epitope tagged receptors, the forms were co-expressed. In samples immunoprecipitated with anti-Myc antibody, anti-HA immunoreactivity was observed following western blotting consistent with the formation of an oligomer. This interaction was shown to be specific as the co-expression of c-Myc tagged muscarinic receptors with the HA tagged β_2 -adrenoceptors resulted in no co-immunoprecipitation observed. This technique has raised concerns as the high levels of receptors expressed and also the hydrophobic nature of the

GPCRs may cause artefactual aggregation resulting in a false positive result. This can be addressed by including controls which represent samples singly expressing the differentially epitope tagged forms of the receptors and mixing prior to immunoprecipitation (Jordan and Devi, 1999).

1.2.1.3 Resonance Energy Transfer (RET) techniques

RET techniques have been extensively used to demonstrate the existence of GPCR oligomers in living cells. These techniques utilise the non-radiative transfer of energy between an energy donor and acceptor pair and are based on the Förster mechanism. Förster theory states that energy transfer efficiency is inversely proportional to the distance between donor and acceptor molecules by the sixth power calculated by;

$$E = 1/(1+[r/(R_0)]^6)$$

(Förster *et al.*, 1948). This demonstrates that energy transfer is highly dependent on the proximity of the donor and acceptor moieties with the permissive distance being <100Å apart to observe significant energy transfer (Wu and Brand, 1994). Receptors under investigation are N- or C-terminally fused with luminescent or fluorescent proteins possessing suitable spectra for use as energy donors or acceptors. Co-expression of both donor and acceptor receptor conjugates and subsequent donor excitation either directly or via bioluminescence, results in energy transfer if donor and acceptor are within RET permissive distance. Energy is re-emitted at a wavelength that is characteristic to the acceptor moiety.

Energy transfer is dependent on the extent of overlap between the donor emission and acceptor excitation spectra. The quantum yield of the donor and the acceptor excitation spectra are also factors effecting energy transfer. The orientation of the donor and acceptor moieties is also crucial and it is possible that an interaction could be occurring that results in the donor and acceptor molecules being organised into an orientation unfavourable to energy transfer (Pfleger and Eidne, 2004; Kroeger and Eidne, 2004).

1.2.1.3.1 Bioluminescence energy transfer (BRET)

BRET is an energy transfer technique based on a bioluminescent donor *Renilla* luciferase (Rluc) and a fluorescent acceptor. This technique was developed from the natural phenomenon of bioluminescence in marine organisms such as the sea pansy *Renilla reniformis* and the jellyfish *Aequora victoria*. Energy transfer is generated by the transfer of energy to a fluorescent acceptor green fluorescent protein (GFP) resulting from the oxidation of the *Renilla* luciferase substrate coelenterazine (Xu *et al.*, 1999). Coelenterazine is a naturally occurring imidazolopyrazine luciferin that can participate in the bioluminescent process. This substrate is cell permeable and several analogs of coelenterazine exist each demonstrating distinct luminescent properties.

The original BRET system utilised Rluc as a donor and a red shifted version of GFP termed enhanced yellow fluorescent protein (eYFP) as the acceptor. The substrate in this technique is the h derivative of coelenterazine. Rluc emits light between 475-480nm while eYFP emits between 525-530nm, resulting in poor spectral resolution. The Rluc produces a broad emission spectrum that overlaps with that of eYFP, creating high 'bleedthrough' of

the Rluc signal into the eYFP window. Therefore the signal: noise ratio of this technique is relatively poor. This methodology was first utilised to detect protein dimerisation for the light-sensitive clock protein KaiB in *E. Coli* (Xu *et al.*, 1999).

These issues have been addressed and a new generation BRET assay has been developed termed BRET². In this assay Rluc is again utilised as the donor, however the substrate used is a coelenterazine derivative termed DeepBlueC (PerkinElmer). This substrate demonstrates distinct spectral properties when oxidised by luciferase and emits light at 395nm. The fluorescent acceptor used is a modified form of GFP termed GFP² which has an excitation spectrum adapted to the emission spectrum of DeepBlueC and upon excitation re-emits energy at 510nm. This allows much higher spectral resolution between donor and acceptor and vastly improving the signal: noise ratios. This technique has been extensively utilised for the detection of protein: protein interactions including the investigation of receptor oligomerisation and also receptor interaction with intracellular proteins including β -arrestin (Mercier *et al.*, 2002; Kroeger *et al.*, 2001; Charest and Bouvier, 2003).

1.2.1.3.2 Fluorescence Resonance Energy Transfer (FRET)

FRET is a technique that utilises fluorescent donor and acceptor proteins with overlapping donor emission and acceptor excitation spectra. Donor excitation is provided by an external light source as opposed to BRET in which excitation was produced via a bioluminescent reaction. Several combinations of donor and acceptor proteins have been used in FRET of which CFP/YFP has been most exploited (Pfleger and Eidne, 2005). Several variations of the FRET technique exist. Excitation of the donor fluorescent moiety

results in the transfer of energy to the acceptor and a corresponding proportional drop in fluorescence intensity of the donor protein. Therefore the difference in donor fluorescence in the absence and presence of the acceptor is a measure of FRET efficiency. Photo bleaching FRET utilises this fact and images of donor fluorescence are compared before and after irreversible photochemical destruction of the acceptor moiety by prolonged sample illumination at a wavelength specific to the acceptor. An increase in donor fluorescence following acceptor destruction demonstrates FRET occurring between donor and acceptor protein conjugates. The technique can also be performed by photo-destruction of the donor fluorescent protein. In this variation, if donor and acceptor are within FRET permissive distance the resulting energy transfer will compete with the photo bleaching process (Pfleger and Eidne, 2005; Zal and Gascoigne, 2004).

Spectral FRET imaging requires three measurements to be recorded in order to measure FRET: sensitised fluorescence and donor and acceptor fluorescence. The close spectral overlaps that exist between the donor and acceptor proteins commonly requires measurements of bleed-through between filter sets to be recorded. FRET is calculated as the ratio of bleed-through corrected sensitised fluorescence to donor or acceptor fluorescence. Fluorescence lifetime imaging (FLIM) is a variation of FRET that exploits the accelerated decay in donor fluorescence caused by FRET following a short pulse of excitation permitting the generation of fluorescence decay profiles.

Time-resolved FRET (Tr-FRET) is a variation that can facilitate the detection of cell surface GPCR oligomers in living cells. Whole cells co-expressing differentially N-terminally epitope tagged receptors are incubated with epitope specific antibodies conjugated to fluorescent moieties capable of participating in energy transfer. As the

antibodies only have access to cell surface expressed GPCRs, this technique will only report energy transfer occurring at the cell membrane. Tr-FRET confers excellent signal to noise ratios. This is achieved by exploiting the finding that in FRET, the lifetime of the acceptor's emission contains a contribution equal to the donor's emission lifetime during energy transfer (Morrison, 1988). Both the donor and acceptor fluorophores commonly used in Tr-FRET display prolonged fluorescence characteristics leading to prolonged emission by the acceptor moiety. Measurements are generally taken following a delay of 50 μ s, allowing the clear distinction of the long-lived acceptor emission signal resulting from FRET and the short lived signal resulting from direct excitation. The fluorescent donor moieties commonly used in this technique are commonly lanthanide compounds such as europium cryptate and europium chelate for which fluorescence is long-lived (Bazin *et al.*, 2002). The acceptor moieties commonly used include allophycocyanin and Alexa Fluor 647 (Pfleger and Eidne, 2005).

1.2.2 GPCR homo- and hetero-oligomers

A vast number of studies have been performed investigating GPCR homo- and hetero-oligomerisation as demonstrated in Tables 1.1 and 1.2. The large number of receptors found to homo-oligomerise within family A GPCRs suggests that this may be a general trend for all members within this subgroup. Hetero-oligomerisation has further added to the complexity of GPCR signalling. Members of the same class of GPCRs have been found to hetero-oligomerise and hetero-oligomers formed of different classes of GPCRs have also been shown.

The majority of studies detailed in Tables 1.1 and 1.2 have utilised systems in which the receptors of interest are not endogenously expressed, commonly using recombinant systems. However, several studies have demonstrated GPCR oligomerisation in cell lines endogenously expressing the receptor of interest. Hetero-oligomer formation has been shown for the angiotensin AT₁ and bradykinin B₂ receptors in rat smooth muscle cells, human platelets and ormental vessels (AbdAlla *et al.*, 2000; AbdAlla *et al.*, 2001). The presence of GABAbR1 and GABAbR2 hetero-oligomers in the rat cortex was demonstrated using receptor specific antibodies for co-immunoprecipitation (Kaupmann *et al.*, 1998). Similarly receptor specific antibodies were used to identify hetero-oligomer formation between the calcium sensing receptor and the glutamate receptors mglu₁ and mglu₅ in the rat brain (Gama *et al.*, 2001). The adenosine A₁ and the purinoceptor P2Y₁ receptors have been found to hetero-oligomerise in membranes obtained from rat brains (Yoshioka *et al.*, 2002).

Recently atomic force microscopy has been used to investigate the organisation of native mouse rhodopsin in the membrane (Fotiadis *et al.*, 2003; Liang *et al.*, 2003). These studies clearly demonstrate the arrangement of rhodopsin receptors in an oligomeric array of closely packed receptor dimers as shown in Figure 1.3a. Individual dimers could be observed that may have broken away from the regimented rows of receptors while occasional monomers were also observed. Although this result may be unique to the rhodopsin system in which the receptor is present at high density, these studies do provide the first conclusive evidence that receptor oligomers exist in native membranes.

1.2.3 Mechanisms of oligomerisation

Studies to determine regions important in GPCR oligomerisation have yielded conflicting results. Initial reports implicated the C-terminal tail region as being important in determining the ability of the delta opioid peptide (DOP) receptor to form oligomers (Cvejic and Devi, 1997). The extracellular N-terminal region was found to be important in bradykinin B₂ receptor oligomer formation (AbdAlla *et al.*, 1999). More recent studies involving the yeast α factor also implicated a role for the N-terminal region (Overton *et al.*, 2002) and a possible role of N-terminal glycosylation in oligomerisation of some class A GPCRs has been shown (Wu *et al.*, 2003). Attention has more recently focused on the potential role of transmembrane domains in oligomerisation. Studies have been performed utilising peptides corresponding to transmembrane regions within receptors. For the β_2 adrenoceptor, dopamine D2 and leukotriene B4 BLT₁ receptors, studies implicated a role for TM VI in that introduction of the peptides corresponding to this region abolished oligomerisation (Herbert *et al.*, 1996; Ng *et al.*, 1996; Baneres and Parello, 2003). However studies utilising peptides must be carefully interpreted as the binding of the peptide to a specific region may prevent oligomerisation not through that region, but by preventing the oligomer forming normally via another region of the receptor (Kroeger and Eidne, 2004). A glycophorin A-like sequence was identified in TM VI of the β_2 adrenoceptor (Lemmon *et al.*, 1994), a finding that initiated the peptide study discussed previously. The importance of this region was demonstrated when a peptide corresponding to the glycophorin A-like sequence was prepared containing three amino acid substitutions. This peptide failed to abolish oligomerisation detected via western blotting (Hebert *et al.*, 1996). A subsequent study identified a similar motif in TM1 of the yeast α factor (Overton *et al.*, 2003), a region previously demonstrated to play a role in the oligomerisation of this

receptor (Overton *et al.*, 2002). Identification of a similar motif in the α_{1B} adrenergic receptor stimulated analogous studies: however two independent reports confirmed that while TM I is important, the glycoporphin A-like motif was not required for receptor homo-oligomerisation (Stanasila *et al.*, 2003; Carrillo *et al.*, 2004). A recent study by Trettel *et al.* (2003), used CXCR2 receptor fragments to demonstrate that homo-oligomerisation of this receptor occurred via a region consisting of extracellular loop 1, TM III and intracellular loop 2. Taken together, this apparently conflicting data gave rise to the notion that oligomerisation for different receptors must occur via different mechanisms.

Many of the techniques currently used cannot distinguish between dimer formation and higher order oligomer formation. This was highlighted in a report implicating TM I, II and IV as being involved in C5a receptor homo-oligomerisation (Klco *et al.*, 2003). These results are difficult to incorporate into a single model of dimer interfaces and the authors concluded that higher order oligomers of this receptor must exist. A recent study investigating rhodopsin in native mouse membranes provided more evidence for this theory. As mentioned previously, rhodopsin was found to exist in an oligomeric array of dimers (Fotiadis *et al.*, 2003). Molecular modelling of the results implicated TM IV and V as potential contact points between monomers forming the dimer, and TM I, II and intracellular loop 3 to be important in providing contact points for oligomer or rows of dimer formation (Liang *et al.*, 2003). This is demonstrated in Figure 1.3b. Further evidence in support of this mechanism of oligomer formation was provided by investigations into the dopamine D2 receptor implicating TM IV as forming a symmetrical dimer interface (Guo *et al.*, 2003; Lee *et al.*, 2003). Hernanz-Falcon *et al.*, (2004) recently demonstrated that single point mutations in TM I and IV of chemokine receptor CCR5 were sufficient to abrogate oligomerisation detected using FRET techniques. A recent study investigating α_{1B}

adrenoceptor homo-oligomer formation utilised data gained from receptor fragments to generate a model of oligomer organisation whereby both TM I and TM IV interactions were involved in symmetrical dimer interactions (Carrillo *et al.*, 2004). They also suggested a possible interaction between TM I + II and TM V + VI receptor fragments, indicating a more complex array of α_{1B} adrenoceptors may form. It is possible that the wide variation in regions found to be important in oligomerisation within a given class of GPCRs could represent different contact points between dimers and higher order oligomers, and a common mechanism could yet be identified.

1.2.4 Regulation of GPCR oligomerisation

1.2.4.1 Effect of ligand on oligomerisation state

There have been several conflicting reports on the effect of ligand on receptor oligomerisation. The main difficulty in this area is the interpretation of any ligand-modulated data as it cannot be ensured that any change in oligomer levels observed are not simply due to ligand binding to pre-formed receptor oligomers changing the conformation of the oligomer and not the ligand actually either promoting oligomer formation or dissociation. Co-immunoprecipitation has been used to investigate the effects of ligand binding on oligomer formation for several receptors and an increase in oligomerisation levels was reported for the β_2 adrenoceptor (Hebert *et al.*, 1996), somatostatin receptor subtypes (Rocheville *et al.*, 2000) and TRH receptors (Zhu *et al.*, 2002). The same technique also reported no change in oligomer levels for the muscarinic M3 receptor (Zeng *et al.*, 1999) and opioid receptors (Jordan and Devi, 1999) and a decrease in reported oligomerisation was observed for the DOP opioid receptor (Cvejic and Devi, 1997) and

dopamine D1 and angiotensin AT₁ hetero-oligomer (Gines *et al.*, 2000). However the technique of co-immunoprecipitation cannot be accurately quantified as changes in the observed immunoreactivity could be a result of ligand binding triggering conformational changes within the oligomer, altering the ability of the antibody to bind the epitope tag, rather than a direct reflection of actual oligomer levels.

RET techniques have also been used to address this issue. RET techniques have demonstrated increases in energy transfer following exposure to agonist for the homo-oligomers: β_2 adrenoceptor (Angers *et al.*, 2000; Cheng *et al.*, 2001), TRH (Krocger *et al.*, 2001), GnRH receptor (Cornea *et al.*, 2001), somatostatin sst₅ receptor (Rocheville *et al.*, 2000), melatonin MT₂ receptor (Ayoub *et al.*, 2002; Terrillon *et al.*, 2003; Ayoub *et al.*, 2004) and the hetero-oligomers adenosine A₁/P2Y₁ (Yoshioka *et al.*, 2002), β_2 adrenoceptors/DOP (McVey *et al.*, 2001), sst₅/dopamine D2 (Rocheville *et al.*, 2000), melatonin MT₁/MT₂ (Ayoub *et al.*, 2002; Ayoub *et al.*, 2004) and TRH₁ and TRH₂ (Hanyaloglu *et al.*, 2002). A decrease in energy transfer was observed for the cholecystokinin CCK₁ receptor (Cheng *et al.*, 2001) and the neuropeptide Y₄ receptor (Berglund *et al.*, 2003). However in the majority of studies performed, authors did not report an alteration in energy transfer following exposure to agonist. This was the case for homo-oligomers of DOP (McVey *et al.*, 2001; Ramsey *et al.*, 2002), Ste2 (Overton *et al.*, 2000), α_{1B} adrenoceptor (Stanasila *et al.*, 2003), complement C5a (Floyd *et al.*, 2003), adenosine A₁ and A_{2A} receptors (Yoshioka *et al.*, 2002; Kamiya *et al.*, 2003; Canals *et al.*, 2004), β_1 adrenoceptor (Mercier *et al.*, 2002; Angers *et al.*, 2000), angiotensin AT₁ (Hansen *et al.*, 2004), calcium sensing receptor (Jensen *et al.*, 2002), cholecystokinin CCK₂ receptor (Cheng *et al.*, 2001), melatonin MT₁ receptor (Ayoub *et al.*, 2002), KOP (Ramsey *et al.*, 2002), oxytocin receptor (Terrillon *et al.*, 2003; Devost *et al.*, 2003) and

the vasopressin V_{1a} and V₂ receptors (Terrillon *et al.*, 2003). Hetero-oligomers studied included adenosine A_{2A} and dopamine D₂ receptors (Canals *et al.*, 2003; Kamiya *et al.*, 2003), β_1 and β_2 adrenoceptors (Lavoie *et al.*, 2002; Mercier *et al.*, 2002), cholecystokinin CCK₁ and CCK₂ (Cheng *et al.*, 2003), MOP and NK₁ receptors (Pfeiffer *et al.*, 2003), oxytocin and vasopressin V_{1a} and V₂ (Terrillon *et al.*, 2003) and vasopressin V_{1a} and V₂ (Terrillon *et al.*, 2003).

The difficulty in interpreting agonist induced changes in energy transfer is the possibility that any modulation observed is in fact a reflection of agonist binding altering the conformation of pre-existing oligomers causing the spatial orientation of the donor and acceptor moieties to change. This is supported by the findings that while agonist binding can cause an increase in BRET for the melatonin MT₁ and MT₂ hetero-oligomer, antagonist binding can also cause the same increase in BRET (Ayoub *et al.*, 2002). This leads to the conclusion that the observed increase is due to receptor occupancy triggering a conformation of donor and acceptor more favourable to energy transfer rather than an increase in oligomer levels.

There have been inconsistencies in the data reported for agonist modulation of oligomers performed using different techniques. Although some reports detailed previously utilising co-immunoprecipitation have demonstrated an increase in oligomerisation following agonist exposure for the β_2 adrenoceptor, RET studies performed by Mercier *et al.*, (2002) have reported no change in energy transfer following agonist exposure. This could be due to a ligand induced conformational change occurring that could not be detected using BRET.

Perhaps the most convincing studies investigating ligand modulation was performed utilising crystallization studies of extracellular ligand-binding domains. These have shown that the mglu₁ receptor (Kunishima *et al.*, 2000) and Wnt-Frizzled (Dann *et al.*, 2001) can form oligomers in the presence and absence of ligand.

1.2.4.2 Site of oligomer formation

Research has demonstrated the existence of oligomers pre-formed at the cell surface. Several studies have addressed the location of oligomer formation. The example of the GABABR1 and GABABR2 obligate dimer discussed previously, illustrates the formation of a hetero-oligomer in the endoplasmic reticulum (ER) and subsequent trafficking of the complex to the cell surface. The exit of GPCRs from the ER represents an important control point whereby incorrectly folded receptors are retained and eventually degraded in the proteosome (Petaja-Repo *et al.*, 2001). Several studies have utilised a cellular fractionation approach in conjunction with energy transfer techniques to demonstrate the presence of oligomers in the ER. The yeast α factor receptor homo-oligomers (Overton *et al.*, 2002), CCR5 homo-oligomers (Issafras *et al.*, 2002) and oxytocin and V₂ vasopressin receptor homo and hetero-oligomers (Terrillon *et al.*, 2003) have all been shown to demonstrate constitutive energy transfer signals in both plasma membrane and ER fractions. Trettel *et al.* (2003) demonstrated that for CXCR2 homo-oligomerisation, oligomers could still be detected in the absence of glycosylated monomeric receptors. This indicates that the receptors oligomerise at some point during the biosynthetic pathway and before they reach the plasma membrane.

The observation that truncated mutants of rhodopsin behave as dominant negative mutants of the wild-type receptor by acting to prevent the normal cell surface expression pattern has further strengthened the hypothesis of oligomer formation in the ER (Colley *et al.*, 1995). Similar studies on the vasopressin V₂ (Zhu and Wess, 1998), dopamine D3 (Karpa *et al.*, 2000), gonadotrophin releasing hormone GnRH (Grosse *et al.*, 1997) and CCR5 chemokine receptors (Benkirane *et al.*, 1997; Shioda *et al.*, 2001) have also yielded similar results.

1.2.5 Functional significance of oligomerisation

Several studies have investigated the effect oligomerisation has on receptor function. Hetero-oligomerisation has been shown to be required for correct signal transduction by the taste receptors. Both sweet and L-amino-acid responses are dependent on the formation of hetero-oligomers between T1R3 and T1R2 or T1R1 respectively (Nelson *et al.*, 2001; Nelson *et al.*, 2002). The idea of a hetero-oligomer possessing distinct pharmacology presents an attractive drug target. A drug could potentially be designed to act as a bifunctional agonist at the hetero-oligomer, stabilising the oligomer, leading to improved signalling (Kcnakin, 2002).

1.2.5.1 Oligomer internalisation

Recently several studies have demonstrated the ability of hetero-oligomers to undergo internalisation in response to an agonist directed at only one receptor in the oligomer complex. This has been shown for somatostatin sst₁ and sst₅ (Rocheville *et al.*, 2000), α_{2A}/β_1 adrenoceptors (Xu *et al.*, 2003), α_{1A}/α_{1B} adrenoceptors (Stanasila *et al.*, 2003),

DOP opioid and β_2 adrenoceptors (Jordan *et al.*, 2001), somatostatin sst₁ and MOP opioid receptors (Pfeiffer *et al.*, 2002) and adenosine A_{2A} and dopamine D2 receptors (Hillion *et al.*, 2002). The hetero-oligomer complex formed between angiotensin AT₁ and bradykinin B₂ demonstrates altered internalisation pathways that are both clathrin and dynamin independent (AbdDalla *et al.*, 2000). In some instances the co-internalisation is accompanied by cross-desensitisation (Pfeiffer *et al.*, 2002; Hillion *et al.*, 2002).

Reports also exist demonstrating receptors that have been shown to respond poorly to agonist induced internalisation can exert a dominant negative effect on a second GPCR that is normally internalised effectively. This has been demonstrated for the KOP receptor that can inhibit the internalisation of the DOP receptor (Jordan and Devi, 1999) and β_2 adrenoceptor (Jordan *et al.*, 2001). This is also the case for the β_1 adrenoceptor that prevented the internalisation of the β_2 adrenoceptor (Lavoie *et al.*, 2002) and the β_3 adrenoceptor that also acted as a dominant negative of the β_2 adrenoceptor (Breit *et al.*, 2004).

1.2.5.2 Pharmacological diversity

Changes in receptor pharmacology have been observed for some hetero-oligomers. The initial observation that hetero-oligomerisation resulted in altered ligand binding affinity was shown for the DOP and KOP opioid receptors (Jordan and Devi, 1999). Co-expression of both receptors resulted in low affinity for ligands specific to either receptor when added separately however high affinity was restored when the agonists were applied together, demonstrating positive co-operativity. Several other examples of co-operativity resulting from hetero-oligomerisation have been shown for α_2 and β_1 adrenoceptors (Werry *et al.*,

2003), muscarinic M2 and M3 (Maggio *et al.*, 1999), DOP and MOP receptors (Gomes *et al.*, 2000), glutamate receptors $mglu_1$ and $mglu_2$ (Galvez *et al.*, 2001), somatostatin sst_3 and dopamine D2 (Rocheville *et al.*, 2000) and adenosine A_{2A} and dopamine D1 (Franco *et al.*, 2000). This finding represents further complexity and diversity in the GPCR field.

Oligomerisation can lead to crosstalk between the monomer units and can result in cooperative binding of ligands to the protomers. An initial radioligand binding study investigating β -adrenergic receptors from frog erythrocytes observed low hill coefficients upon ligand binding and interpreted this as evidence for negative cooperativity (Limbird *et al.*, 1975). Positive cooperativity has been reported for ligand binding of the DOP and KOP receptors upon co-expression (Jordan and Devi, 1999). Negative cooperativity has been observed for D2 dopamine receptor agonist binding in the presence of an adenosine A_2 receptor agonist when the two receptors are co-expressed (Franco *et al.*, 2000). Negative cooperativity has also been observed in the binding of antagonists to the M2 muscarinic and the D2 dopamine receptors for which an antagonist for one receptor appeared to inhibit another in a non-competitive manner (Park *et al.*, 2002; Armstrong and Strange, 2001). Complex mathematical analysis of the results indicated that the data were consistent with cooperativity existing between two or four interacting sites for the D2 dopamine and M2 muscarinic receptor respectively. Cooperative effects have also been observed for homo-oligomers. Cooperativity in the binding of oxytocin to its receptor has been reported in frog bladder epithelial cells (Roy *et al.*, 1973).

Traditionally it has been assumed that a single heterotrimeric G protein interacts with a single receptor monomer. The discovery of GPCR oligomerisation has challenged this view leading to the hypothesis of a receptor dimer interacting with a single G protein.

Analysis of the crystal structure of the heterotrimeric G-protein has suggested multiple contact points between the receptor and the G protein α and $\beta\gamma$ subunits (Hamm, 2001), inconsistent with a one receptor: one G protein stoichiometry. When the X-ray crystal structure of rhodopsin was resolved, these contact points could be investigated relative to the structure. This revealed that the cytoplasmic surface of rhodopsin is no more than 40Å across, a surface too small to accommodate interactions between a monomer receptor and both α and $\beta\gamma$ subunits simultaneously (Hamm, 2001, Liang *et al.*, 2003). Also functional studies have investigated cooperativity of binding of transducin to rhodopsin, yielding data again consistent with a receptor dimer interacting with a single G protein (Willardson *et al.*, 1993; Clark *et al.*, 2001). These studies led to the conclusion that two monomers must bind a single G protein. Baneres and Parello (2003) investigated the interaction between the leukotriene B₄ BLT₁ receptor and the G $\alpha_{12}\beta_1\gamma_2$ heterotrimer. Utilising chemical cross linking followed by size-exclusion chromatography, mass spectroscopy and neutron scattering measurements, this study demonstrated that the complex formed between the activated receptor and G protein in cells corresponds to one GPCR dimer interacting with a single heterotrimeric G protein.

1.3 Chemokines

Chemokines are a large family of chemotactic cytokines. Chemokines and their receptors are involved in several physiological processes including leukocyte trafficking, organogenesis, hematopoiesis and immuno-modulation (Baggiolini, 1998). They have been implicated in several disease states including inflammation and allergy and are crucial in mediating innate and adaptive immune responses. They are small mostly basic secretory

proteins of 8 -12kD with four conserved cysteines (Baggiolini *et al.*, 1994; Baggiolini *et al.*, 1997) produced by leucocytes and tissue cells.

There are two main subfamilies of chemokines; CXC and CC chemokines that are determined by the position of the first two cysteines. In the CXC subfamily the cysteines are separated by one amino acid while in the CC subgroup the cysteine residues are adjacent. These residues also determine the three-dimensional folding of the chemokines by forming two disulphide bonds (Cys1 → Cys3; Cys2 → Cys4). Chemokines generally have a short amino-terminal domain of between 3-10 amino acids preceding the first cysteine and possess a backbone made of β -strands and the connecting loops formed between the second and fourth cysteines. There is a carboxyl terminal α -helix of 20-30 amino acids. The backbone structure is very rigid while the terminal regions display a high degree of structural disorder (Rajaratnam *et al.*, 1995). Throughout the chemokine family there exists similar folding despite highly divergent sequence identity.

Other chemokine family members have been identified which do not belong to either subfamily. Lymphotactin has been identified, possessing only two conserved cysteine residues (Kennedy *et al.*, 1995) and fractalkine, which is membrane bound and possesses three amino acids between the first two cysteine residues.

There are thought to be as many as 40-50 human chemokines, detailed in Figure 1.4. The genes encoding these chemokines have been mapped and found to cluster at specific loci in accordance with the subfamily they are classified into. Generally the genes corresponding to CC chemokines are grouped at 17q11.2-12 and CXC chemokines at 4q21.1. However, some exceptions exist to this rule. CCL17 and CCL22 are found at gene locus 16q13 while

CCL19, CCL21, CCL25 and CCL27 are found at locus 9p13.3. CCL24 and CCL26 are found at 7q11.23 while CCL20 is found at 2q36.3. CXCL12 is found at 10q11.21 and CXCL13 is found at 5q31.1 (Baggiolini, 2001)

CXC- chemokines can be further subdivided on the basis of possessing a three amino acid 'ELR' motif located between the N-terminus and the first cysteine (Clark-Lewis *et al.*, 1991; Hebert *et al.*, 1991). This glutamate – leucine – arginine motif has been found in several chemokines that possess potent neutrophil chemoattractant properties. One member of this subgroup is Interleukin-8 (IL-8) also termed CXCL8 (Yoshimura *et al.*, 1987). IL-8 regulates the recruitment and activation of polymorphonuclear leukocytes (PMNs) at inflammatory sites. Interestingly, IL-8 has been demonstrated to be present in solution as both monomers and dimers, in equilibrium under physiological conditions (Horchler *et al.*, 1998). However data has suggested that while the monomer is capable of binding the CXCR1 receptor, the dimeric form is not. The existence of both forms in normal physiology has been hypothesised to be a negative regulator in receptor function and positive regulator for binding to cell surface glycosaminoglycans (GAGs) (Fernando *et al.*, 2004). GAGs are important in the immobilization of chemokines and have an important role in governing their local concentration.

1.3.1 Chemokine Receptors

The effects of chemokines on target cells are mediated via interactions with seven transmembrane receptors that are G-protein coupled (Murphy, 1994). Six receptors have been identified that bind the CXC- class of chemokines, termed CXCR1-6, while the CC receptor family comprises ten known receptors, CCR1 to CCR10 as detailed in Figure 1.4.

Receptors for fractalkine and lymphotactin have also been identified. Receptors termed Duffy antigen receptor for chemokines (DARC) and D6 have also been identified (Horuk *et al.*, 1993; Nibbs *et al.*, 1997). Although these receptors are hepta-helical chemokine binding proteins they have not been demonstrated to signal therefore were excluded from the nomenclature (Murphy *et al.*, 2000).

Chemokine receptors have 25 – 80% amino acid sequence homology, demonstrating the diversity of the family. Chemokine receptors are generally 340-370 amino acids in length. The extracellular amino terminus is short, commonly possesses N-linked glycoylation sites and may be sulphated on tyrosine residues. There are seven α -helical transmembrane domains with three intracellular and three extracellular connecting loops composed of hydrophilic amino acid residues. The second intracellular loop demonstrates a highly conserved DRYLAIV sequence while the third intracellular loop is short and basic. The extracellular loops 1 and 2 are linked via the formation of a disulphide bond between highly conserved cysteines. The intracellular carboxyl terminal contains serine and threonine residues that act as phosphorylation sites, important in receptor regulation (Murdoch and Finn, 2000).

Currently 18 different chemokine receptors have been identified (Murphy *et al.*, 2000). As mentioned earlier over 50 human chemokines have been identified suggesting a high degree of redundancy within the family whereby several chemokines may bind one receptor within a given subgroup. The finding that several chemokines possess both agonist and antagonist properties under physiological conditions further complicates these interactions. This is the case for the CXCR3 agonists CXCL11, CXCL9 and CXCL10, implicated in T helper 1 cell mediated responses. These agonists have been shown to act as

antagonists at CCR3, a receptor involved in mediating T helper 2 cell type responses (Loetscher *et al.*, 2000). This has also been demonstrated for the CCR3 agonist CCL11 that is a partial agonist at CCR2 and CCR5 (Martinelli *et al.*, 2001; Olgilvie *et al.*, 2001) and CCL7 that acts as an agonist at CCR1, CCR2 and CCR3 but as an antagonist for CCR5 (Blanplain *et al.*, 1999). This adds further complexity to interpreting chemokine signalling.

Chemokines interact with their receptors via the N-terminal region and the exposed conformationally rigid loop of the backbone between the second and third cysteine (Clark-Lewis *et al.*, 1995). These sites are in close proximity due to the formation of the disulphide bonds. It is thought that the receptor recognises the loop region of the chemokine initially and this interaction is crucial for correct presentation of the 'triggering' N-terminal domain to the receptor (Clark-Lewis *et al.*, 1995). The importance of the N-terminal region is highlighted in studies whereby modification of this region has resulted in receptor antagonists (Baggiolini *et al.*, 1994; Zhang *et al.*, 1994; Simmons *et al.*, 1997). The chemokine receptors have been found to generate Pertussis toxin sensitive responses indicating coupling to G α_i proteins.

Chemokine receptors range from being vital, in the case of CXCR4 required for late embryogenesis and organogenesis, to being redundant, demonstrated by CCR5 that is missing in some populations to no obvious detrimental effect (Murphy *et al.*, 2000).

1.3.3 CXCR1 and CXCR2

In 1991 two groups identified the receptors for IL-8 termed CXCR1 (Holmes *et al.*, 1991) and CXCR2 (Murphy *et al.*, 1991). CXCR1 is encoded by 350 amino acids and has an N-

linked glycosylation site at the N-terminus. CXCR2 has a very high sequence homology to CXCR1 (78%) having almost identical intracellular loops. The main divergence occurs at the amino- and carboxyl- terminal (Wu *et al.*, 1993). Both CXCR1 and CXCR2 bind ELR containing CXC chemokines including IL-8 that binds both receptors with high affinity. The genes encoding CXCR1 and CXCR2, as well as a pseudogene for CXCR2, are located on human chromosome 2q35 located 20kb apart (Ahuja *et al.*, 1992; White *et al.*, 1994).

The expression patterns of the two receptors have been determined using receptor specific monoclonal antibodies. It was shown that CXCR1 and CXCR2 are expressed on all granulocytes, monocytes and mast cells. Some CD8+ T-cells and CD56+ natural killer cells also express the receptors (Chuntharapai *et al.*, 1994). Neutrophils were found to express equal amounts of CXCR1 and CXCR2 however monocytes and positive lymphocytes express more CXCR2 than CXCR1 (Chuntharapai *et al.*, 1994). CXCR2 expression has been detected in some cell types in the Central nervous system (CNS). Functional CXCR2 was detected in Purkinje cells in human brain slices, foetal neurons and in a human neuronal cell line, hNT, binding IL-8 with affinity in the nano-molar range (Horuk *et al.*, 1997; Hesselgeser *et al.*, 1997). Interestingly, to date CXCR1 expression has not been demonstrated in the CNS.

CXCR2 will bind all ELR containing CXC chemokines with close affinity values observed. CXCR1 is highly selective for IL-8 with a greater than 50-fold difference in affinity (Lee *et al.*, 1992; Loetscher *et al.*, 1994; Ahuja and Murphy, 1996). GCP-2 (CXCL6) is equipotent at CXCR1 and CXCR2 (Wuyts *et al.*, 1997). CXCR2 can bind all other ELR containing chemokines including GRO- α (CXCL1), NAP-2 (CXCL7) and ENA-78 (CXCL5). The N-terminal region of the two receptors has been shown to

determine the ligand specificity. A study performed by LaRosa *et al.*, (1992) constructed a series of hybrid receptors in which the N-terminus of one receptor was exchanged for the other. This demonstrated that when CXCR1 had the CXCR2 amino terminus it could bind both IL-8 and GRO α , however the converse hybrid in which CXCR2 was transplanted with the N-terminal region of CXCR1 retained the ability to bind IL-8 but was unable to bind GRO α . These results highlight the importance of the N-terminal region of the receptor in determining the specificity of ligand binding. The same study also identified two residues present in the third extracellular loop as being critical for ligand binding.

A knockout model for CXCR2 has been generated in mice. CXCR2 (-/-) animals display a lack of ability of mobilise neutrophils in response to chemical peritoneal challenge and neutrophils were unable to respond chemotactically to chemokine challenge directly at CXCR2. The animals also demonstrated lymphadenopathy, caused by an increased level of B cells, and splenomegaly, resulting from an increase in metamyelocytes and mature neutrophils (Cacalano *et al.*, 1994; Moore *et al.*, 1995). However, it should be noted that the mouse model is a poor one as there is no murine equivalent of IL-8 and a mouse equivalent of CXCR1 has not been identified.

CXCR1 and CXCR2 have been reported to vary significantly in their ability to activate signal transduction pathways. In neutrophils, both IL-8 and GRO- α can induce calcium mobilization and calcium release (Walz *et al.*, 1991; Schroder *et al.*, 1990), however only IL-8 can activate phospholipase-D and the formation of superoxide by NADPH oxidase (L'Heureux *et al.*, 1995). Calcium mobilization also appears to be mediated via different mechanism for the two receptors. IL-8 induced calcium release occurs only from intracellular stores while GRO- α exposure causes influx of extracellular calcium as well as

release of calcium from intracellular stores (Damaj *et al.*, 1996). In neutrophils it has been hypothesised that the major receptor for IL-8 is CXCR1. Hammond *et al.* (1995) utilised antibodies selective for CXCR1 or CXCR2 capable of preventing agonist binding. The anti-CXCR2 antibody was found to almost completely abolish chemotaxis induced by GRO- α while having only a slight effect on IL-8 induced mobilisation. The anti-CXCR1 antibody was shown to completely abrogate chemotaxis induced by IL-8 and only weakly effect GRO- α mediated responses.

CXCR1 and CXCR2 are rapidly desensitised following exposure to agonist and undergo substantial internalisation. Desensitisation is mediated by phosphorylation of serine and threonine residues present on the C-terminal tail of CXCR1 and CXCR2 (Richardson *et al.*, 1995; Mueller *et al.*, 1997; Ben-Baruch *et al.*, 1997; Richardson *et al.*, 1998). CXCR1 is capable of generating signals for cross-phosphorylation and cross-desensitisation however CXCR2 is not. This is thought to be dependent on the duration of action as CXCR2 is rapidly phosphorylated terminating signalling (Richardson *et al.*, 2003). Chuntharapai *et al.*, (1995) demonstrated that IL-8 induced down-modulation of the CXCR1 and CXCR2 by >90% in human neutrophils within five minutes of ligand exposure. However differences in receptor recycling have been observed with differing cell type studied. In neutrophils, which endogenously express CXCR1 and CXCR2, CXCR1 was rapidly recycled to the cell surface with full expression restored 1.5 hours following agonist exposure. However three hours following agonist exposure CXCR2 was still only partially restored to previous levels (Chuntharapai *et al.*, 1995). A substantial amount of CXCR2 was sequestered in early and late endosomal vesicles of neutrophils. In epithelial cells both receptors were demonstrated to recycle with similar kinetics (Fan *et al.*, 2003; Samanta *et al.*, 1990). The reason for these divergent results could be due to the

existence of cross-regulatory signals existing in neutrophils where both CXCR1 and CXCR2 are expressed rather than in heterologous systems such as epithelial cells which may only express one receptor in isolation. Studies performed in HEK cells have demonstrated areas important in receptor internalisation. For CXCR1 this was found to be an area in the C terminal tail which contains four clusters of serine and threonine residues (Prado *et al.*, 1996; Richardson *et al.*, 1995) while for CXCR2 an LLKIL motif present in the C terminal tail has been shown to be important in internalisation (Fan *et al.*, 2001).

Internalisation of CXCR1 and CXCR2 occurs via β -arrestin coupled pathways involving interactions with dynamin and AP-2 and sequestration into clathrin-coated pits (Barlic *et al.*, 1999; Yang *et al.*, 1999; Fan *et al.*, 2001). However, β -arrestin complex formation is not necessary for CXCR2 sequestration as removal of CXCR2 from the cell membrane has been observed even for truncated forms of CXCR2 that cannot interact with β -arrestin (Fan *et al.*, 2001; Zhao *et al.*, 2004). This arrestin independent internalisation is thought to be mediated via an alternative pathway thought to be dynamin/Rac dependent (Schlunck *et al.*, 2004).

As mentioned previously, unlike CXCR1, CXCR2 cannot stimulate the respiratory burst response. This has recently been attributed to a protective effect of β -arrestin inhibiting the respiratory burst in cells expressing CXCR2. Zhao *et al.*, (2004) demonstrated that in β -arrestin knock out cells there is enhanced MAPK activation resulting from the action of reactive oxygen species whose formation is normally inhibited by β -arrestin. Under physiological conditions it is thought that the rapid phosphorylation of CXCR2, occurring within 15 seconds, and complex formation with β -arrestin supersedes that of NADPH oxidase, preventing the respiratory burst from occurring (Zhao *et al.*, 2004). This

represents an important control mechanism in co-ordinating cell responses to bacterial infection. β -arrestin has also been implicated in initiating signalling cascades. Following ligand activation CXCR1 associates with β -arrestin. This interaction has been shown to recruit the Src family members Hck and c-Fgr to the receptor- β -arrestin complex. Hck becomes activated and the β -arrestin-Hck complex can migrate to granules, initiating granule fusion and content release. Cells expressing a dominant negative form of β -arrestin did not release granules following exposure to IL-8, illustrating the importance of this β -arrestin mediated signal (Barlic *et al.*, 2000).

Responses to IL-8 and GRO- α in neutrophils are markedly inhibited by Pertussis toxin (PTx), suggesting coupling to the G_i family of G proteins (Bacon and Camp, 1990; Thelen *et al.*, 1988; Wuyts *et al.*, 1997). Physical association of CXCR1 and CXCR2 to $G\alpha_{i2}$ has been demonstrated (Damaj *et al.*, 1996). Four residues located within intracellular loop 2 and one residue in intracellular loop 3 have been implicated as crucial for calcium signalling and have been identified as putative residues involved in G-protein coupling (Damaj *et al.*, 1996). However, some studies have observed a small element of PTx insensitive signalling suggesting that CXCR1 and CXCR2 may be able to couple to other PTx insensitive G proteins such as G_q (Wu *et al.*, 1996; Hall *et al.*, 1999). Interestingly chemokine induced chemotaxis is a PTx sensitive response suggesting coupling to $G\alpha_i$ proteins. However, chemotaxis has been shown to be mediated via $G\beta\gamma$ subunits and $G\alpha_i$ is not required for cell migration induced by IL-8 stimulation of CXCR1 (Neptune *et al.*, 1999). Studies have highlighted a correlation between RGS protein expression and an inhibition of chemokine induced chemotaxis (Bowman *et al.*, 1998; Reif *et al.*, 2000). CXCR2 has been shown to specifically interact with the PDZ domain of RGS12 (Snow *et al.*, 1998).

Recently Rose *et al.*, (2004) determined that IL-8 could induce maximal chemotaxis and calcium flux responses at concentrations 10-fold less than those required for receptor endocytosis. Also CXCR1 and CXCR2 could be re-activated by the same ligand at a concentration sufficient to induce signalling but too low to stimulate receptor sequestration. This suggests a role for receptor internalisation as an end point in CXCR1 signalling when the cell has reached the site of inflammation and the chemokine concentration is at its highest (Rose *et al.*, 2004).

1.3.3 Chemokine receptor oligomerisation

Several conflicting reports exist in the area of chemokine receptor oligomerisation. Initial studies indicated that chemokine receptor oligomerisation may be analogous to that of cytokine receptor oligomerisation, which is ligand dependent. Oligomerisation of CCR2 was demonstrated using both immunoprecipitation and antibody fragment cross-linking to illustrate the existence of agonist dependent CCR2 oligomers (Rodriguez-Frade *et al.*, 1999). This study also utilised a mutant form of CCR2 that is unable to signal and demonstrated that upon co-expression with wild type CCR2, normal function was abrogated and immunoprecipitation data showed a complex formed between the mutant and wild type receptors. Ligand dependent oligomerisation was also shown for CCR5 and CXCR4 homo-oligomers and hetero-oligomer formation between CCR5 and CCR2 (Vila-Coro *et al.*, 2000; Vila-Coro *et al.*, 1999; Mellado *et al.*, 2001). Hetero-oligomer formation was also found to confer unique signalling properties to the receptors involved. This included G protein switching from $G\alpha_i$ to $G\alpha_{q/11}$, suggested by a reduced threshold of agonist required to trigger PTx-resistant calcium flux. The activation kinetics of

phosphoinositide 3-kinase (PI 3-K) are distinct from that observed for homo-oligomers and cell adhesion is preferentially activated in contrast to cell migration presented for homo-oligomers. Interestingly hetero-oligomer formation was found to prevent internalisation and desensitisation to a second agonist stimulus (Mellado *et al.*, 2001).

However, subsequent studies utilising energy transfer technique and, interestingly, co-immunoprecipitation demonstrated the existence of constitutive chemokine receptor oligomers. This is the case for CXCR2, CXCR4 and CCR5 homo-oligomers (Trettel *et al.*, 2003; Babcock *et al.*, 2003; Issafras *et al.*, 2002; Toth *et al.*, 2004; Hernanz-Falcon *et al.*, 2004; Percherancier *et al.*, 2005). In studies investigating the effect of ligand binding on energy transfer for CXCR4 and CCR5, Toth *et al.*, (2004) and Hernanz-Falcon *et al.*, (2004) demonstrated a significant elevation of RET signal upon ligand binding for CXCR4 and CCR5 respectively. For CXCR4, binding of the CXCR4 antagonist AMD3100 was able to attenuate the ligand induced increase in energy transfer (Toth *et al.*, 2004). The difficulty in interpretation of ligand modulation of oligomer formation has been discussed previously however the authors hypothesized that chemokine oligomers may exist in equilibrium between monomeric and dimeric units and ligand binding may influence this delicate balance (Toth *et al.*, 2004). The varied results in this field can be somewhat explained by variation in the technique sensitivity however it is evident that more research is required to elucidate the role of ligand in chemokine oligomerisation.

1.3.4 Chemokines as therapeutic targets

Attention was drawn to the possibility of chemokine receptors as potential drug targets upon the discovery that CCR5 and CXCR4 act as co-receptors for HIV-1 entry into cells

(Littman, 1998; Hoffman and Doms, 1998). Infection with HIV-1 involves two interactions with cell surface proteins. The initial interaction is between the cell surface protein CD4 and viral protein gp120, triggering a conformational change permitting interaction with the chemokine receptor. During initial infection this receptor is CCR5 however, as HIV progresses to AIDS, CXCR4 dependent strains emerge. Chemokine receptor interaction induces a second conformational change exposing the peptide gp41 that fuses with the target cell membrane (Proudfoot, 2002). Chemokines have also been implicated in the metastasis of several types of cancers especially breast cancer where increased levels of CXCR4 have been demonstrated in tumours (Kang *et al.*, 2003). Muller *et al.*, (2001) demonstrated that a specific monoclonal antibody against CXCR4 could inhibit metastasis of breast cancer cells to the lung or lymph nodes in mice. Multiple sclerosis (MS) is an autoimmune disease in which chemokine receptors are central to pathogenesis. Excessive levels of CCR1, CCR5 and CXCR3 have been demonstrated in brain samples derived from MS patients (Sorensen *et al.*, 1999; Balashov *et al.*, 1999).

CXCR1 and CXCR2 have been implicated in several inflammation disease states. This can be explained as these receptors are potent mediators of chemotaxis and inflammation is the result of excessive recruitment of leukocytes. CXCR1 and CXCR2 have been implicated in airway diseases such as chronic obstructive pulmonary disease (COPD) and severe asthma where the inflammation pathology is mediated by neutrophils (Owen, 2001). IL-8 has been implicated in the pathology of several conditions including psoriasis (Nickoloff *et al.*, 1991; Bizzarri *et al.*, 2003), ulcerative colitis (Mahida *et al.*, 1992) and cystic fibrosis (Bizzarri *et al.*, 2003). GlaxoSmithKline has developed a low molecular weight antagonist to CXCR2. This has been shown to inhibit both rabbit and human neutrophil chemotaxis mediated by both IL-8 and GRO- α *in vitro*. *In vivo* experiments performed in rabbits

demonstrated the ability of the antagonist to inhibit chemotaxis in response to IL-8 (White *et al.*, 1999). A separate study utilising a non-peptide antagonist against CXCR2 in a rabbit arthritis model demonstrated reduced accumulation of neutrophils, monocytes and lymphocytes in the synovial fluid (Podolin *et al.*, 2002). An IL-8 specific monoclonal antibody has been used in clinical trials for the treatment of the inflammatory skin disease psoriasis (Yang *et al.*, 1999). While CXCR1 and CXCR2 have not been shown to act as co-receptors for HIV-1 (D'Souza *et al.*, 2000), a role exists in HIV replication. Blockade of CXCR1 and CXCR2 has been shown to inhibit HIV-1 replication in both T lymphocytes and macrophages (Lane *et al.*, 2001). This is thought to be due to HIV-1 infection in a cell stimulating IL-8 and GRO- α production that can then act to increase HIV-1 replication. Increased levels of IL-8 have been found in the lymphoid tissues of patients with AIDS. A non-competitive allosteric small-molecule inhibitor of CXCR1 and CXCR2 termed Reperixin has been identified as a possible therapeutic agent to combat reperfusion injury (RI) that is unavoidable in organ transplant (Bertini *et al.*, 2004). RI also factors in the pathology of several other conditions including myocardial infarction and stroke. This molecule acts by locking CXCR1 and CXCR2 in an inactive conformation, preventing receptor signalling and PMN chemotaxis. Granule release and pro-inflammatory cytokine production was also inhibited (Casilli *et al.*, 2005). Reperixin has proven safe in animal studies and phase I trials performed in human volunteers have demonstrated the drug to be well tolerated. All small molecule inhibitors of IL-8 currently described are still in the pre-clinical phase (Casilli *et al.*, 2005)

CXCR1 and CXCR2 are involved in cancer pathology. IL-8 is constitutively produced by melanoma cells but not by untransformed melanocytes (Dhawan and Richmond, 2002). ELR+ chemokines including IL-8 have been demonstrated to mediate angiogenesis and

tumorigenesis during the development of ovarian carcinoma (Yoneda *et al.*, 1998). Increased expression of CXCR2 by melanoma cells has also been demonstrated and CXC chemokines can increase survival, proliferation and tumour cell migration (Dhawan and Richmond, 2002). CXCR2 has been implicated in cellular transformation with the similarity between CXCR2 and the viral GPCR encoded in Kaposi's sarcoma herpes virus (KSHV) that signal constitutively. Over expression of KSHV GPCR can result in lesions analogous to those observed for Kaposi's sarcoma (Yang *et al.*, 2000).

1.4 Project aims

The aim of this study was to investigate CXCR1 and CXCR2 receptor homo- and hetero-oligomerisation. This was examined utilising several techniques including co-immunoprecipitation, BRET, Tr-FRET and FRET. The selectivity of oligomerisation was examined by investigating interactions with an unrelated GPCR. The effect of ligand on oligomerisation state was investigated, as was the site of oligomer formation. The overlapping expression pattern of CXCR1 and CXCR2 with members of the opioid receptor family has been demonstrated. Investigation into potential interactions between the receptors has been performed in this study.

Figure 1.1 Diversity of G protein coupled receptor (GPCR) signalling

GPCRs can be activated by a wide variety of ligands that trigger G protein dependent and independent signalling pathways. These pathways can influence many biological responses. (Adapted from Marinissen and Gutkind, 2001)

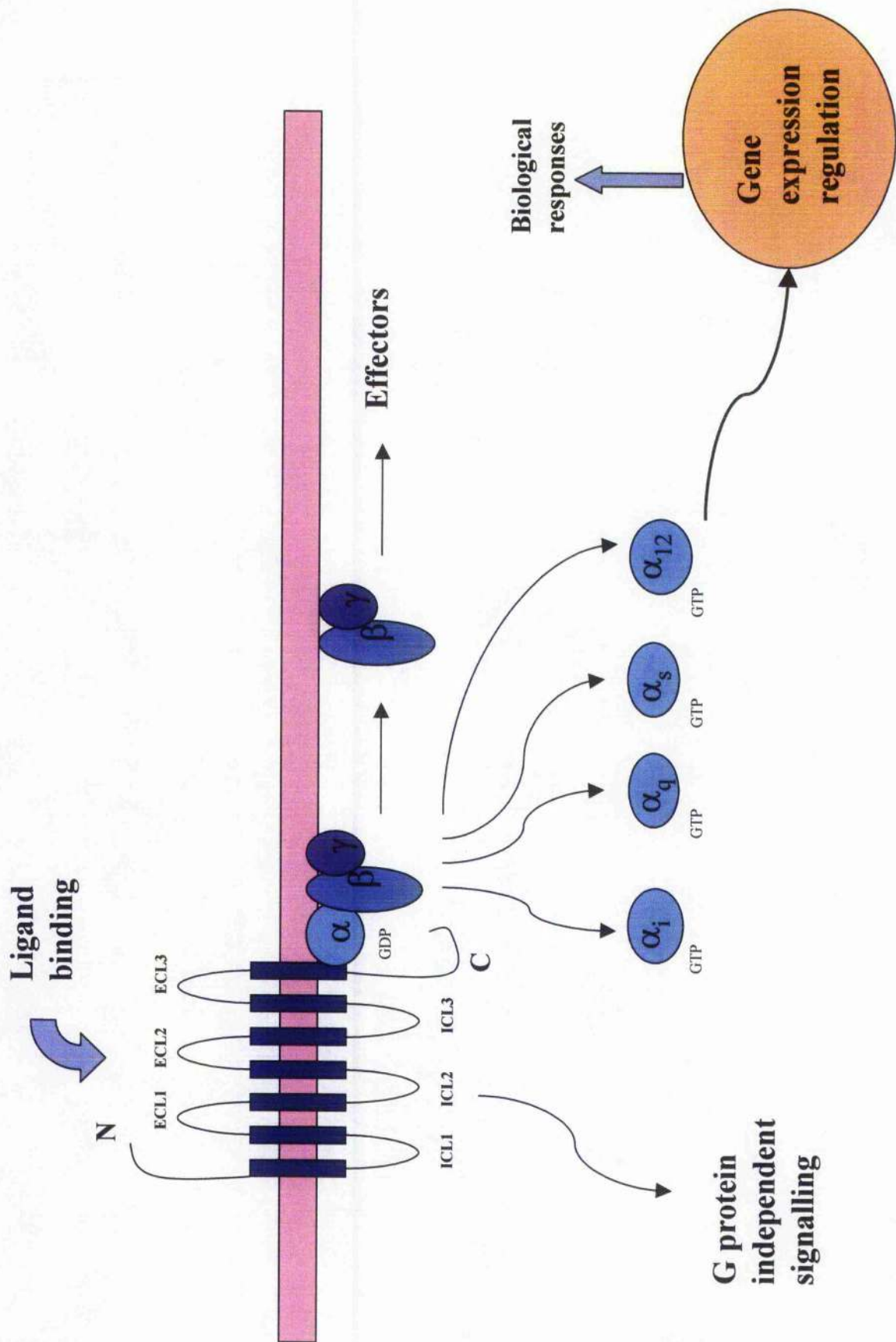
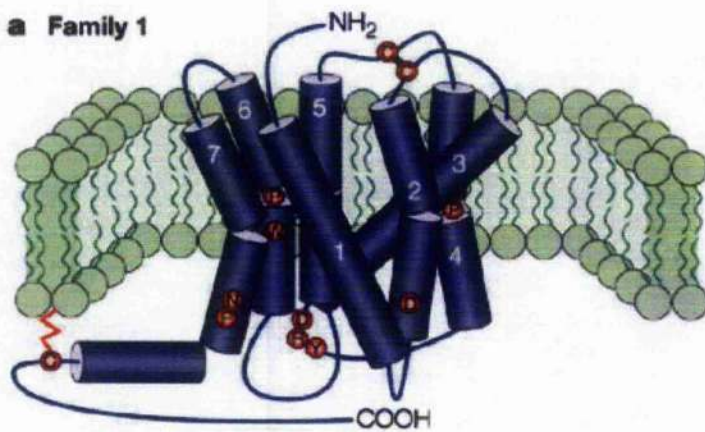


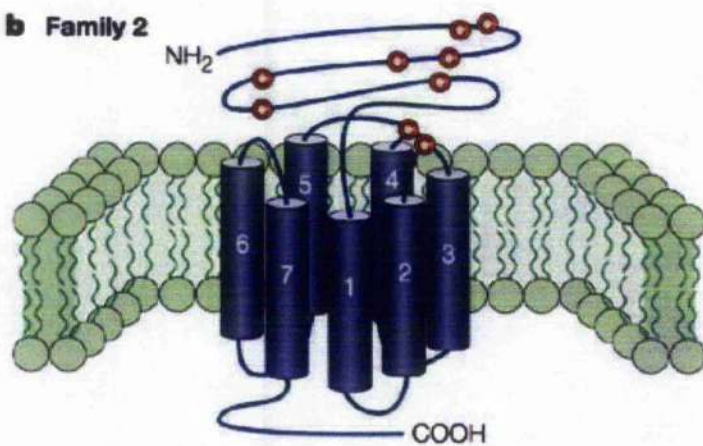
Figure 1.2 Structural classification of GPCRs

GPCRs can be divided into subgroups according to both sequence similarity and pharmacological nature of ligand. Schematic representations of the three main sub-families are shown, illustrating the main structural determinants unique to each sub-group. (Taken from George *et al.*, 2002)

a Family 1



b Family 2



c Family 3

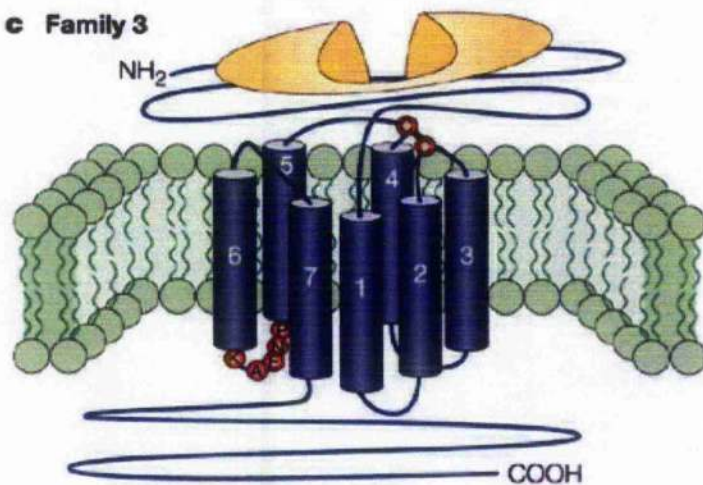
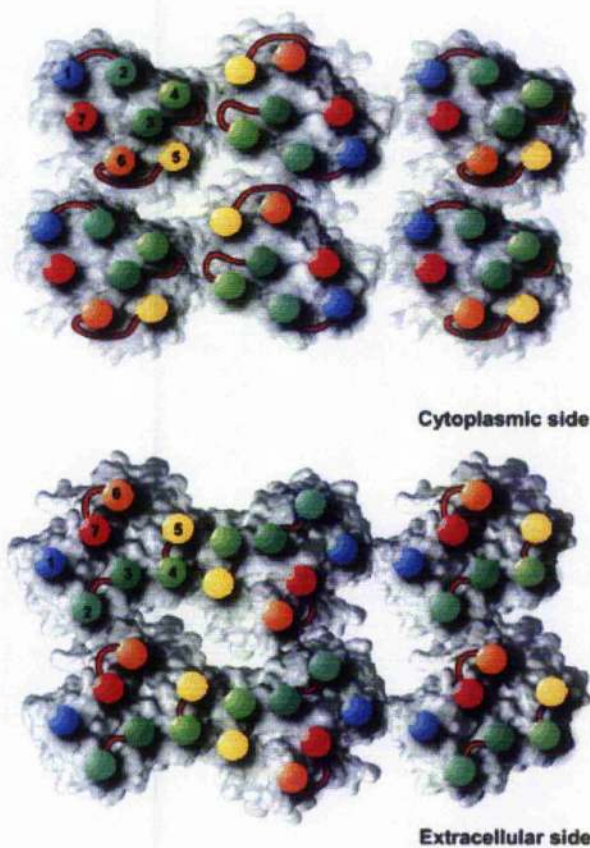


Figure 1.3 Organisation of the cytoplasmic surface of rhodopsin

Atomic-force microscopy was used to investigate rhodopsin organisation in the native disc membrane. The topograph image shown in (a) demonstrates a paracrystalline arrangement of rhodopsin dimers. Rhodopsin dimers are circled while arrows show receptor monomers. A model for the packing arrangement of rhodopsin molecules within the array is also displayed (b). The contacts between monomers are predicted to be formed by the transmembrane regions IV and V shown in yellow-green and yellow respectively. ((a) taken from Fotiadis *et al.*, 2003 and (b) taken from Fotiadis *et al.*, 2004)



(a)



(b)

Table 1.1 GPCR homo-oligomer formation

Several examples of GPCR homo-oligomerisation have been demonstrated using a variety of techniques.

GPCR	Technique	Reference
α_{1A} -adrenoceptor	Immunoprecipitation, FRET	Stanasila <i>et al.</i> , 2003
β_1 -adrenoceptor	BRET	Lavoie <i>et al.</i> , 2002; Mercier <i>et al.</i> , 2002
α_{1B} -adrenoceptor	Immunoprecipitation, FRET, Tr-FRET	Stanasila <i>et al.</i> , 2003; Carrillo <i>et al.</i> , 2003
β_2 -adrenoceptor	Immunoprecipitation, BRET	Angers <i>et al.</i> , 2000; Hebert <i>et al.</i> , 1996; Lavoie <i>et al.</i> , 2002; McVey <i>et al.</i> , 2001; Mercier <i>et al.</i> , 2002; Hebert <i>et al.</i> , 1998
β_3 -adrenergic receptor	BRET	Breit <i>et al.</i> , 2004
Adenosine A ₁	BRET	Yoshioka <i>et al.</i> , 2002
Adenosine A _{2A}	BRET, FRET	Kamiya <i>et al.</i> , 2003; Canals <i>et al.</i> , 2004
Angiotensin AT ₁	BRET	Hansen <i>et al.</i> , 2004
C5a receptor	FRET	Floyd <i>et al.</i> , 2003
Calcium-sensing receptor	Immunoprecipitation, BRET	Bai <i>et al.</i> , 1999; Jensen <i>et al.</i> , 2001
CCR2	Immunoprecipitation	Rodriguez-Frade <i>et al.</i> , 1999
CCR5	Immunoprecipitation, BRET	Vila-Coro <i>et al.</i> , 2000; Issafras <i>et al.</i> , 2002
Cholecystokinin receptor CCK ₁	BRET	Cheng <i>et al.</i> , 2001
Cholecystokinin receptor CCK ₂	BRET	Cheng <i>et al.</i> , 2003
CXCR2	Immunoprecipitation	Trettel <i>et al.</i> , 2003
CXCR4	BRET, FRET	Babcock <i>et al.</i> , 2003; Issafras <i>et al.</i> , 2002; Toth <i>et al.</i> , 2004
D1 dopamine receptor	Immunoprecipitation	George <i>et al.</i> , 1998
D2 dopamine receptor	Immunoprecipitation, FRET	Lee <i>et al.</i> , 2000; Wurch <i>et al.</i> , 2001
DOP receptor	Immunoprecipitation, Tr-FRET, BRET	Cveijic <i>et al.</i> , 1997; George <i>et al.</i> , 2000; Gomes <i>et al.</i> , 2002; McVey <i>et al.</i> , 2001
Glutamate mglu ₁	Immunoprecipitation	Robbins <i>et al.</i> , 1999
Glutamate mglu ₅	Immunoprecipitation	Romano <i>et al.</i> , 1996
GnRH receptor	Photobleaching FRET, BRET	Cornea <i>et al.</i> , 2001; Horvat <i>et al.</i> , 2001; Kroeger <i>et al.</i> , 2001
KOP receptor	Immunoprecipitation	Jordan <i>et al.</i> , 1999
LH receptor	Photobleaching FRET	Roess <i>et al.</i> , 2000
Melatonin MT ₁ receptor	Immunoprecipitation, BRET	Ayoub <i>et al.</i> , 2002; Ayoub <i>et al.</i> , 2004
Melatonin MT ₂ receptor	Immunoprecipitation	Ayoub <i>et al.</i> , 2002; Ayoub <i>et al.</i> , 2004; Terrillon <i>et al.</i> , 2003
MOP receptor	Immunoprecipitation, BRET	George <i>et al.</i> , 2000; Gomes <i>et al.</i> , 2002
Neuropeptide Y ₄	BRET	Berglund <i>et al.</i> , 2003
Oxytocin	BRET	Terrillon <i>et al.</i> , 2003; Devost <i>et al.</i> , 2003; Devost <i>et al.</i> ,

		2004
Somatostatin sst ₅	Immunoprecipitation, FRET	Rocheville <i>et al.</i> , 2000
Thyrotropin receptor	FRET	Latif <i>et al.</i> , 2002
TRH ₁	Immunoprecipitation, BRET	Hanyaloglu <i>et al.</i> , 2002; Kroeger <i>et al.</i> , 2001; Zhu <i>et al.</i> , 2002
TRH ₂	BRET	Hanyaloglu <i>et al.</i> , 2002
Vasopressin V _{1a}	BRET	Terillon <i>et al.</i> , 2003
Vasopressin V ₂	BRET	Terillon <i>et al.</i> , 2003
Yeast α factor receptor	FRET	Overton <i>et al.</i> , 2000, Overton <i>et al.</i> , 2002

Table 1.2 GPCR hetero-oligomer formation

Hetero-oligomerisation between closely related and distinct GPCRs has been shown. These hetero-oligomers can form between members of the same receptor class or between receptor classes.

GPCR	Technique	Reference
Class A : Class A		
β_1 and β_2 -adrenoceptors	Immunoprecipitation, BRET	Lavoie <i>et al.</i> , 2002; Mercier <i>et al.</i> , 2002
α_{1A} -adrenoceptor and α_{1B} -adrenoceptor	FRET	Stanasila <i>et al.</i> , 2003
β_2 and β_3 -adrenoceptors	BRET	Breit <i>et al.</i> , 2004
α_{2A} -adrenoceptor and β_1 -adrenoceptor	Immunoprecipitation	Xu <i>et al.</i> , 2003
β_2 -adrenoceptor and DOP	Immunoprecipitation, BRET, Tr-FRET	McVey <i>et al.</i> , 2001; Jordan <i>et al.</i> , 2001
β_2 -adrenoceptor and KOP	Immunoprecipitation	Jordan <i>et al.</i> , 2001
5-HT _{1B} and 5-HT _{1D}	Immunoprecipitation	Xie <i>et al.</i> , 1999
Adenosine A ₁ and Dopamine D1 receptor	Immunoprecipitation	Gines <i>et al.</i> , 2000
Adenosine A ₁ and P2Y ₁	Immunoprecipitation, BRET	Yoshioka <i>et al.</i> , 2002; Yoshioka <i>et al.</i> , 2002
Adenosine A _{2A} and dopamine D2 receptor	Immunoprecipitation, BRET	Hillion <i>et al.</i> , 2002; Kamiya <i>et al.</i> , 2003
Angiotensin AT _{1A} and AT ₂ receptor	Immunoprecipitation	AbdAlla <i>et al.</i> , 2001
Angiotensin AT _{1A} and Bradykinin B ₂ receptor	Immunoprecipitation	AbdAlla <i>et al.</i> , 2000
CCR2 and CCR5	Immunoprecipitation, FRET, BRET	Mellado <i>et al.</i> , 2001; El-Asmar <i>et al.</i> , 2005
Cholecystokinin CCK ₁ and CCK ₂	BRET	Cheng <i>et al.</i> , 2003
D2 and D3 dopamine receptor	Functional reconstitution	Scarselli <i>et al.</i> , 2001
D2 dopamine receptor and somatostatin sst ₅	Photo-bleaching FRET	Rocheville <i>et al.</i> , 2000
KOP and DOP	Immunoprecipitation, BRET	Jordan <i>et al.</i> , 1999; Gomes <i>et al.</i> , 2002
Melatonin MT ₁ and M ₂	Immunoprecipitation, BRET	Ayoub <i>et al.</i> , 2002
MOP and DOP	Immunoprecipitation	George <i>et al.</i> , 2000; Gomes <i>et al.</i> , 2000
MOP and NK ₁	BRET	Pfeiffer <i>et al.</i> , 2003
Muscarinic M3 and α_{2A} -adrenoceptor	Chimeric receptor reconstitution	Maggio <i>et al.</i> , 1993
Muscarinic M2 and M3	Receptor reconstitution	Maggio <i>et al.</i> , 1999
Oxytocin and Vasopressin V _{1a}	BRET	Terillon <i>et al.</i> , 2003
Oxytocin and Vasopressin V ₂	BRET	Terillon <i>et al.</i> , 2003
Somatostatin sst ₁ and sst ₅	Photo-bleaching FRET	Rocheville <i>et al.</i> , 2000
Somatostatin sstr ₁ and MOP	Immunoprecipitation	Pfeiffer <i>et al.</i> , 2002
Somatostatin sstr ₁ and sstr ₃	Immunoprecipitation	Pfeiffer <i>et al.</i> , 2001
TRH ₁ and TRH ₂	BRET	Hanyaloglu <i>et al.</i> , 2002
Vasopressin V _{1a} and V ₂	BRET	Terillon <i>et al.</i> , 2003
Class A : Class C		
5-HT _{1A} and GABA _B R2	Immunoprecipitation	Salim <i>et al.</i> , 2002
Adenosine A ₁ and glutamate	Immunoprecipitation	Ciruela <i>et al.</i> , 2001

Adenosine A_{2A} and glutamate mglu ₁ mglu ₅	Immunoprecipitation	Ferre <i>et al.</i> , 2002
Class C : Class C		
Calcium sensing receptor and glutamate mglu ₁	Immunoprecipitation	Gama <i>et al.</i> , 2001
Calcium sensing receptor and glutamate mglu ₅	Immunoprecipitation	Gama <i>et al.</i> , 2001
GABAbR1 and GABAbR2	Immunoprecipitation	Jones <i>et al.</i> , 1998; Kaupmann <i>et al.</i> , 1998; White <i>et al.</i> , 1998

Figure 1.4 Chemokine receptors and their ligands

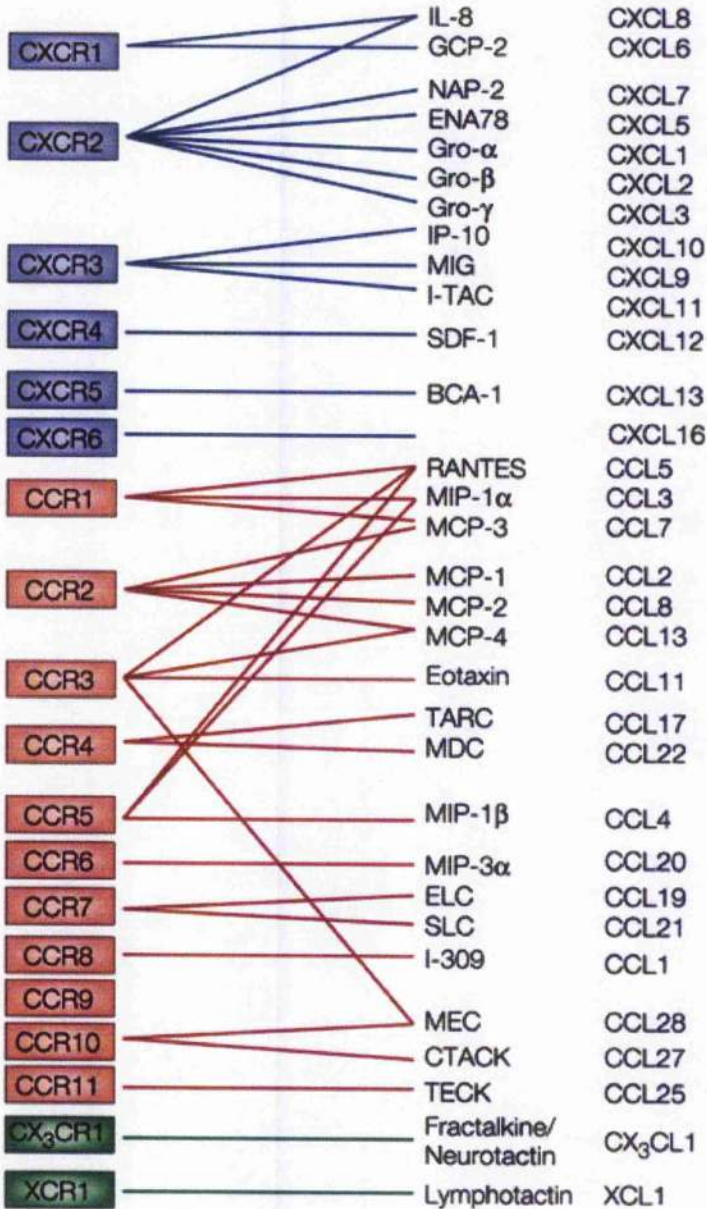
Chemokine receptors are divided in subfamilies in accordance with the position of N-terminal cysteine residues. The CXC- receptors are detailed in blue, CC- receptors are shown in red and the minor subclass comprising C- and CX3C- receptors in green. Receptor ligands are detailed as both common names and systematic names following adoption of a systematic nomenclature system. (Taken from Proudfoot, 2002)

Chemokine receptors

Chemokines

Common names

Systematic names



2.0 Materials and Methods

2.1 Materials

2.1.1 General reagents, enzymes and kits

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK

Rainbow markers

BDH, Lutterworth, Leicestershire, UK

22mm coverslips, microscope slides, sodium di-hydrogen orthophosphate, potassium hydroxide, potassium chloride, methanol, isopropanol

Cell Signalling Technology, Beverly, MA, USA

Polyclonal anti-c-Myc antibody

Chemicon International, Science Centre, Hampshire, UK

ReBlot plus solution

Duchefa, Haarlem, The Netherlands

Yeast extract, tryptone, agar

Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK

Glycine, HEPES, sucrose, SDS, potassium di-hydrogen orthophosphate, calcium chloride, manganese chloride

Interactiva, Ulm, Germany

All oligonucleotides used for PCR reactions

Invitrogen BV, Groningen, The Netherlands

NuPage Novex pre-cast 8-12% Bis-Tris gels, MOPS running buffer

Konica Europe, Hohenbrunn, Germany

X-ray film

PerkinElmer

DeepBlueC, 384 black walled plates, Optiplate white 96 well plates

Pierce, Perbio Science UK Ltd., Tattenhall, Cheshire, UK

Supersignal West Pico chemiluminescent substrate

Promega UK Ltd., Southampton, UK

All restriction endonucleases, T4 DNA ligase, Pfu polymerase, Wizard Plus SV minipreps

Quiagen, Crawley, West Sussex, UK

QiaQuick PCR purification kit, QiaQuick gel extraction kit, Qiafilter maxi-prep kit.

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete EDTA-free protease inhibitor tablets, 1 Kb DNA ladder

R&D systems, Abingdon, UK

Human recombinant IL-8 and Gro- α

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Magnesium chloride, sodium chloride, sodium hydroxide, sodium acetate, DTT, di-sodium orthophosphate, tris base, EDTA, bromophenol blue, deoxycholic acid (sodium salt), Hoechst stain, rubidium chloride, Triton X-100, DMSO, glycerol, tween 20, ethylene glycol, paraformaldehyde, ampicillin, ethidium bromide, IBMX, forskolin

Whatman International Ltd., Maidstone, UK

GF/C Glass filters

2.1.2 Tissue culture plasticware and reagents

American Tissue Culture Collection, Rockville, USA

HEK293T cells

Costar, Cambridge, MA., USA

5mL, 10mL and 25mL. pipettes, 75cm² and 125cm² vented tissue culture flasks, 6 well plates, 100cm² dishes

Invitrogen BV, Groningen, The Netherlands

Lipofectamine transfection reagent, Optimem-1, L-glutamine (200mM)

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

DMEM, 0.25% trypsin-EDTA, poly-D-Lysine.

2.1.3 Radiochemicals

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK

^{125}I IL-8

2.1.4 Antisera

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK

Donkey anti-mouse IgG-HRP conjugate, donkey anti-rabbit IgG-HRP conjugate

Cell Signalling Technologies

Anti-Myc polyclonal antibody

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Anti-Flag M2 antibody

Roche Diagnostics Ltd., Lewes, East Sussex, UK

12CA5 monoclonal antibody (binds to haemagglutinin epitope tagged proteins), anti-VSV antibody

Molecular Probes, Eugene, Oregon, USA

Anti-mouse Alexa® 594 IgG conjugate, anti-mouse Alexa® 488 IgG conjugate, anti-rabbit Alexa® 488 IgG conjugate

2.2 Buffers

2.2.1 General Buffers

Phosphate Buffered Saline (10x)

137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 , 10.2mM Na_2HPO_4 , dissolved in 1L H_2O , pH 7.4. The solution was autoclaved prior to use. 1xPBS was prepared by diluting the stock 1:10 in H_2O .

Tris-EDTA (TE) Buffer

10mM Tris base, 0.1mM EDTA, pH 7.4

Laemmli Buffer (2x)

0.4M DTT, 0.17M SDS, 50mM Tris, 5M urea, 0.01% (w/v) bromophenol blue

2.2.2 Molecular Biology solutions

TAE Buffer (50x)

40mM Tris, 1mM EDTA, glacial acetic acid. This solution was diluted 1:50 prior to use.

DNA loading Buffer (6x)

0.25% bromophenol blue, 40% sucrose (w/v) in H₂O

LB Media (Luria-Bertani Medium)

10g Bacto-Tryptone, 5g Yeast Extract and 10g sodium chloride dissolved in H₂O up to 1L and pH 7.4. The medium was autoclaved prior to use.

Buffer 1 (for preparation of competent bacteria)

100mL of this solution was prepared containing; 3mL 1M potassium acetate, 1mL 1M rubidium chloride, 1mL 1M calcium chloride, 5mL manganese chloride and 18.75mL 80% (w/v) glycerol. The solution was adjusted to pH 5.8 and the volume corrected to 100mL in H₂O. The solution was filter sterilised and stored at 4°C.

Buffer 2 (for preparation of competent bacteria)

40mL of this solution was prepared containing: 4mL 100mM MOPS pH 6.5, 3mL 1M calcium chloride, 0.4mL 1M rubidium chloride, 7.5mL 80%(w/v) glycerol. The pH was corrected to 6.5 and the volume adjusted to 40mL using H₂O. The solution was filter sterilised and stored at 4°C until use.

2.3 Molecular Biology Protocols

2.3.1 LB agar plates

LB was prepared as detailed previously. 15g/L of bacto-agar was added to the LB and autoclaved. On removal from the autoclave, the bottle was gently inverted to distribute the

agar throughout the solution. The mix was cooled to 50°C prior to addition of an antibiotic. The final concentration of antibiotic used were; ampicillin – 100µg/mL or Zeocin – 50µg/mL. The medium was mixed gently and approximately 25 mL poured into 10cm² petri dishes. The plates were left to set at room temperature before being stored at 4°C. Unused plates were disposed of three weeks following preparation.

2.3.2 Preparation of competent bacteria

Competent bacteria are cells that have been treated to enable them to accept plasmids following transformation. A scraping of DH5α cells was taken from lab stocks, streaked out onto a minimal agar plate and incubated at 37°C overnight. A single colony was selected, inoculated into a 5mL culture of L-Broth and grown in a shaking incubator for 16 hours. This culture was then sub-cultured into 100mL L-Broth and grown in a shaking incubator at 37°C until the optical density at 550nm was 0.48 – approximately 90 minutes of incubation. The culture was then chilled on ice for 5 minutes before being centrifuged at 1811 x g for 10 minutes at 4°C. All traces of L-Broth were removed and then the pellet was resuspended in 20mL of Buffer 1 (as detailed above) by gently pipetting up and down. The suspension was incubated on ice for 5 minutes prior to being centrifuged as before. The pellet was resuspended in 2 mL of buffer 2 (as detailed above) by pipetting and the suspension incubated on ice for 15 minutes. The cells were then divided into 220µL aliquots and stored at -80°C until required.

2.3.3 Transformation

An aliquot of competent bacteria was thawed on ice and 50µL of cells per transformation

reaction aliquoted into a sterile tube. Between 1 and 10ng of DNA was then added to the cells and incubated on ice for 15 minutes. The cells were then subjected to a heat shock at 42°C for 90 seconds and then returned to ice for 2 minutes. 450µL of L-Broth was added to the cells and the mix incubated for 45 minutes at 37°C in a shaking incubator. 200µL of the mix was plated onto an LB plate containing the appropriate antibiotic and incubated inverted for 12-16 hours at 37°C.

2.3.4 Preparation of plasmid DNA

2.3.4.1 Mini-preps

Mini-prep cDNA was purified from bacterial cultures using the Promega SV minipreps kit. 5mL of bacterial culture was centrifuged at 16.1 x g for 10 minutes and the supernatant removed. The pellet was resuspended in 250µL of cell resuspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/mL Rnase A) immediately followed by 250µL of lysis buffer (0.2 NaOH, 1% SDS). 10µL of alkaline phosphatase was added to each sample and incubated for 5 minutes. 350µL of neutralising buffer was then added (4.09M guanidine hydrochloride, 0.76M potassium acetate, 2.12M glacial acetic acid, pH 4.2) to precipitate the bacterial chromosomal DNA. This was centrifuged for 10 minutes at 16.1 x g and the supernatant applied to a DNA purification column. The column was centrifuged briefly to bind the DNA and the column washed twice in wash buffer (60mM potassium acetate, 10mM Tris-HCl pH 7.4, 60% ethanol). DNA was eluted by adding 100µL of sterile water.

2.3.4.2 Maxi-preps

The Qiagen QIAfilter kit was used to produce larger scale DNA samples. As detailed previously, 5mL of bacterial culture was grown up overnight and this culture used to inoculate a 100mL LB culture. This was incubated for 16-18 hours at 37°C in a shaking incubator. Bacteria were pelleted by centrifugation for 30 minutes at 3220 x g at 4°C. The pellet was then resuspended in 10mL of chilled buffer P1 (50mM Tris-HCl pH8.0, 10mM EDTA, 100µg/mL Rnase A) by vortexing and the cells lysed by adding 10mL of buffer P2 (200mM NaOH, 1% SDS) and incubating for 10 minutes at room temperature. 10mL of buffer P3 (3.0M potassium acetate pH 5.5) was added to neutralise the reaction and the solution immediately applied to a QIAfilter cartridge. This was left for 10 minutes at room temperature to incubate. During this time a QIAGEN tip 500 was equilibrated by adding 10mL of buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol). The plunger was then inserted into the cartridge and the lysate filtered into the equilibrated tip. The column was washed with 60mL of buffer QC (1.0M NaCl, 50mM MOPS pH 7.0, 15% isopropanol). The DNA was eluted by adding 15mL of buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% isopropanol). The DNA was precipitated by the addition of 10.5mL isopropanol and the mixture centrifuged at 27216 x g for 30 minutes in a cooled centrifuge. The pellet was washed in 5ml of room temperature 70% ethanol and again centrifuged for 15 minutes at 27216 x g. The supernatant was carefully removed and the pellet allowed to air dry prior to being re-suspended in 1mL of sterile water.

2.3.5 DNA quantification

Quantification of DNA samples prepared was performed by examining the absorbance of a

1:200 dilution of the sample at 260nm. An A_{260} value of 1 unit was taken to correspond to 50 μ g /mL of double stranded DNA.

2.3.6 Digestion of DNA with restriction endonucleases

Restriction digests of DNA were performed in order to prepare for sub-cloning of PCR fragments or to ensure the successful ligation of a construct. Digests were prepared in a final volume of 20 μ L using Promega enzymes as directed for each individual enzyme. Reactions were incubated at 37°C for 2 hours before being examined using DNA gel electrophoresis.

2.3.7 DNA gel electrophoresis

Digested DNA samples were examined using gel electrophoresis to ensure correct ligation of a construct. This method was also used to isolate plasmid vector. Samples were mixed with 2X loading buffer. A 1% (w/v) agarose gel was prepared by mixing agarose with 1X TAE buffer and heating until the agarose had melted. 2.5mg/mL ethidium bromide was added to the gel and then poured into horizontal gel tanks. The gel was left to set and then 1X TAE buffer added. The sample was then loaded onto the gel and ran at 75mA until the samples were well resolved. The DNA fragments were visualised using ultraviolet light. The size of the fragments was assessed by comparison with a 1Kb DNA ladder.

2.3.8 DNA purification from agarose gels

DNA was purified from the gel by excising the band of interest and using the QiaQuick gel

extraction kit in accordance with manufacturers instructions. Briefly, the DNA-gel fragment was dissolved in buffer QG by heating at 50°C. One gel volume of isopropanol was added to the mix and the solution transferred to a QiaQuick column. The DNA was bound to the column by centrifugation. The sample was washed using buffer PE supplemented with ethanol and then eluted from the column using sterile water.

2.3.9 DNA ligations

Constructs were generated by ligating digested PCR fragments into plasmid vector using T4 DNA ligase. For each construct a ratio of 1:3 and 1:6 vector:PCR product was used in a volume of 20µL. 1µL of T4 ligase was used with 2µL of ligase buffer and the reaction incubated at 4°C for at least 16 hours. The ligation reactions were transformed as detailed in section 2.3.3.

2.3.10 Polymerase Chain Reaction

PCR reactions were established in a volume of 50µL containing 10ng of template DNA, 0.2mM dNTPs (dATP, dCTP, dGTP, dTTP), 25pmol of sense and anti-sense oligonucleotide primers, 1x Pfu polymerase buffer and 1 unit of Pfu polymerase enzyme. Reactions were carried out on an Eppendorf gradient Thermocycler. PCR cycles used were;

<i>Denaturation</i>	<i>Annealing</i>	<i>Extension</i>	<i>Cycles</i>
95°C, 1 min	50-60°C, 1 min	72°C, 2 min	30
95°C, 1 min	50-60°C, 1 min	72°C, 2 min	1

2.4 Generation of CXCR1 constructs

Wild type human CXCR1 obtained from AstraZeneca was used as a PCR template for all CXCR1 constructs.

2.4.1 N-terminal tagged constructs

Flag-CXCR1

Primers encoding the Flag epitope sequence were used to generate N-terminally tagged Flag-CXCR1 and introduce a stop codon;

Sense 5'-

AAAAGAATTCGCCACCATGGACTACAAGGACGACGATGATAAGTCAAATATTA
CAGATCCAC – 3'

Anti-sense 5' – AAAAGAATTCTCAGAGGTTGGAAGAGACATTGAC – 3'.

The region encoding the Flag epitope tag is shown in bold italics. The *EcoRI* site is underlined and the amplified fragment digested and ligated into pcDNA3.

c-Myc-CXCR1

Primers encoding the c-MYC epitope sequence were used to generate N-terminally tagged c-Myc-CXCR1 and introduce a stop codon;

Sense 5' --

AAAAGAATTCGCCACCATGGAACAAAAACTTATTCTGAAGAAGATCTGTCAA
TATTACAGATCCAC – 3'

Anti-sense 5' - AAAAGAATTCTCAGAGGTTGGAAGAGACATTGAC – 3'.

The region encoding the c-Myc epitope is shown in bold italics. The *EcoRI* site is underlined and the amplified fragment digested and ligated into pcDNA3.

HA-CXCR1

Primers encoding the HA epitope sequence were used to generate N-terminally tagged HA-CXCR1 and introduce a stop codon;

Sense5' –

AAAAGGTACCGCCACCATGTATCCCTACGACGTCCCCGATTATGCGTCAAATAT
TACAGATCCAC – 3'

Anti-sense 5' - AAAAGAATTCTCAGAGGTTGGAAGAGACATTGAC – 3'.

The region encoding the HA epitope tag is shown in bold italics. The *KpnI* site present in the sense primer and the *EcoRI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3.

HA-CXCR1-ER

Primers encoding the HA epitope sequence were used to introduce an N-terminally tagged HA sequence and remove the stop codon;

Sense 5' –

AAAAGGTACCGCCACCATGTATCCCTACGACGTCCCCGATTATGCGTCAAATAT
TACAGATCCAC – 3'

Anti-sense 5' – AAAAGAATTCTCAGAGGTTGGAAGAGACATTGAC – 3'.

The region encoding the HA epitope tag is shown in bold italics. The *KpnI* site present in the sense primer and the *EcoRI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3.

Primers encoding the ER sequence (KHILFRRRRRGFRQ) were annealed;

Sense 5' –

AATTCAAGCATATCCTCTTTCGAAGGAGGAGAAGGGGCTTCAGGCAATGAT –

3',

Anti-sense 5'

CTAGATCATTGCCTGAAGCCCCTTCTCCTCCTTCGAAAGAGGATATGCTTG – 3'.

The *Eco*RI present in the sense primer and the *Xba*I site present in the anti-sense primer are underlined and the fragment digested and ligated downstream of the HA-CXCR1 fragment in frame in pcDNA3.

2.4.2 C-terminal tagged constructs

CXCR1GFP²

Primers were designed to amplify the CXCR1 receptor and remove the stop codon;

Sense 5' – AAAAGAATTCGCCACCATGTCAAATATTACAGATCCAC-3',

Anti-sense 5' – AAAAGGTACCGAGGTTGGAAGAGACATTGAC – 3'. The *Eco*RI and *Kpn*I sites present in the sense and anti-sense primers respectively are shown underlined.

The amplified fragment was digested and ligated into pGFP²-N2 in frame with GFP².

CXCR1Rluc

Primers were designed to amplify CXCR1 and remove the stop codon;

Sense 5' – AAAA AAGCTTGCCACCATGTCAAATATTACAGATCCAC – 3',

Anti-sense 5' – AAAACTCGAGGTTGGAAGAGACATTGAC – 3'. The *Hind*III and *Xho*I sites present in the sense and anti-sense primers respectively are shown underlined.

The amplified fragment was digested and ligated into pcDNA3 upstream and in frame with

Renilla Luciferase ligated between *Xho*I and *Xba*I.

CXCR1YFP

Primers were designed to amplify CXCR1 and remove the stop codon;

Sense 5' - AAAA AAGCTTGCCACCATGTCAAATATTACAGATCCAC - 3', Anti-sense 5' - AAAAGCGGCCGCGAGGTTGGAAGAGACATTGAC - 3'. The *Hind*III and *Not*I sites encoded in the sense and anti-sense primers are underlined. The amplified fragment was digested and ligated into pcDNA3.1 (+) upstream and in frame with eYFP ligated between *Not*I and *Xho*I.

CXCR1CFP

Primers were designed to amplify CXCR1 and remove the stop codon;

Sense 5' - AAAA AAGCTTGCCACCATGTCAAATATTACAGATCCAC - 3', Anti-sense 5' - AAAAAGGTACCGAGGTTGGAAGAGACATTGAC - 3'. The *Hind*III and *Kpn*I sites encoded in the sense and anti-sense primers are underlined. The amplified fragment was digested and ligated into pcDNA3 upstream and in frame with cCFP ligated between *Hind*III and *Xho*I.

2.5 Generation of CXCR2 constructs

Wild type human CXCR2 obtained from AstraZeneca was used as a PCR template for all CXCR2 constructs.

2.5.1 N-terminal tagged constructs

Flag-CXCR2

Primers encoding the Flag epitope sequence were used to generate N-terminally tagged Flag-CXCR2 and introduce a stop codon;

Sense 5' -

AAAAGAATTTCGCCACCATGG***ACTACAAGGACGACGATGATAAGGAAGATTTTA***
ACATGGAG - 3',

Anti-sense 5' - AAAAGAATTTCGAGAGTGGAAGTGTGCCC - 3'.

The region encoding the Flag epitope tag is shown in bold italics. The *EcoRI* site present in both sense and anti-sense primers is underlined and the amplified fragment was digested and ligated into pcDNA3.

c-Myc-CXCR2

Primers encoding the c-Myc epitope sequence were used to generate N-terminally tagged c-Myc-CXCR2 and introduce a stop codon;

Sense 5' -

AAAAGAATTTCGCCACCATGG***AACAAAACTTATTTCTGAAGAAGATCTGGAAGA***
TTTAAACATGGAG - 3',

Anti-sense 5' - AAAAGAATTTCGAGAGTGGAAGTGTGCCC - 3'.

The region encoding the c-Myc epitope tag is shown in bold italics. The *EcoRI* site is underlined and the amplified fragment was digested and ligated into pcDNA3.

VSV-CXCR2

Primers encoding the VSV epitope sequence were used to generate N-terminally tagged

VSV-CXCR2 and introduce a stop codon;

Sense 5' -

AAAAGGGTACCGCCACCATGTACACCGACATCGAAATGAACCGCCTTGGTAAGG
AAGATTTTAACATGGAG- 3',

anti-sense 5' - AAAAGAATTCGAGAGTGGAAGTGTGCCC - 3'.

The region encoding the VSV epitope tag is shown in bold italics. The *KpnI* and *EcoRI* sites present in the sense and anti-sense primers respectively are underlined and the amplified fragment was digested and ligated into pcDNA3.

2.5.2 C-terminal tagged constructs

CXCR2GFP²

Primes were designed to amplify CXCR2 and remove the stop codon;

Sense 5' - AAAAGAATTCGCCACCATGGAAGATTTTAACATGGAC - 3', antisense
5' - AAAAGGTACCGAGAGTAGTGGAAGTGTGCCC - 3'.

The *EcoRI* and *KpnI* present in the sense and anti-sense primers respectively are underlined. The amplified fragment was digested and ligated into pGFP²-N2 in frame with GFP².

CXCR2Rluc

Primers were designed to amplify CXCR2 and remove the stop codon;

Sense 5' - AAAAAGCTTGCCACCATGGAAGATTTTAACATGGAG - 3',

Anti-sense 5' - AAAACTCGAGGAGCGTCGTGGAAGTGTG - 3'. The *HindIII* and *XhoI* sites present in the sense and anti-sense primers respectively are shown underlined.

The amplified fragment was digested and ligated into pcDNA3 upstream and in frame with

Renilla Luciferase ligated between *Xho*I and *Xba*I.

CXCR2YFP

Primers were designed to amplify CXCR2 and remove the stop codon;

Sense 5' - AAAAAGCTTGCCACCATGGAAGATTTTAACATGGAG - 3', Anti-sense

5' - AAAAGCGGCCGCAGAGTAGTGGAAGTGTGCCC - 3'. The *Hind*III

and *Not*I sites encoded in the sense and anti-sense primers are underlined. The amplified fragment was digested and ligated into pcDNA3.1 (+) upstream and in frame with eYFP ligated between *Not*I and *Xho*I.

CXCR2CFP

Primers were designed to amplify CXCR2 and remove the stop codon;

Sense 5' - AAAAAGCTTGCCACCATGGAAGATTTTAACATGGAG - 3',

Anti-sense 5' - AAAAAGGTACCGAGAGTAGTGGAAGTGTGCCC - 3'.

The *Hind*III and *Kpn*I sites encoded in the sense and anti-sense primers are underlined. The amplified fragment was digested and ligated into pcDNA3 upstream and in frame with eCFP ligated between *Hind*III and *Xho*I.

2.6 Routine cell culture

2.6.1 Cell maintenance

Human embryonic kidney cells (HEK293T) were used in this study. The cells were grown in 75cm² flasks in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and 2mM L-Glutamine (DMEM/NBCS). The cells were maintained in a humidified incubator of 95% air / 5% CO₂ at 37°C.

2.6.2 Cell subculture

Once confluent, cells were sub-cultured by the addition of sterile 0.25% Trypsin-EDTA solution. The medium was removed from the flask and 2mL of trypsin solution added. The flask was gently rotated to cover the monolayer and replaced in the incubator for 3 minutes. Once the cells had detached, 3mL of fresh medium was added to the flask and the cells resuspended by gently pipetting. The cells were then sub-cultured into either 75cm² flasks to maintain the cell line or 10cm² dishes for transfection.

2.6.3 Transient transfection

Transfection of HEK293T cells was performed when the cells had reached 60-70% confluency. Plasmid DNA was transfected using Lipofectamine in accordance with the manufacturers instructions. Plasmid DNA was diluted to 0.1µg/µL and the volume corresponding to the amount of DNA to be transfected was transferred to a 15mL tissue culture tube. A volume of Optimem was added in order to bring the volume in the tube to 600µL. A mix of Lipofectamine™ and Optimem™ was prepared in the ratio of 20µL Lipofectamine and 580µL Optimem for each sample and 600µL of the mix added gently to the DNA mix and incubated for 30 minutes. During this time the cells were washed with Optimem. Following the incubation period, 4.8mL of Optimem was added to the sample tubes and the mix transferred onto the cells. The cells were returned to the incubator for 4-5 hours before removing the transfection mix and replacing with DMEM/NBCS media. The media was changed 24 hours later and the cells harvested approximately 48 hours

post-transfection. A similar protocol was used for transfection in a six-well plate however the ratio of Lipofectamine to Optimem was 1:29.

2.6.4 Cell harvesting

Transfected cells were harvested 48 hours post-transfection. The media was removed and the cells washed 3 times in 1 X PBS. Cells were scraped off the flask/dish in 5mL of 1 X PBS using a cell scraper and transferred to a 15mL centrifuge tube. The flask/dish was washed again in 5mL 1 X PBS to remove any additional cells, before centrifuging the harvested cells for 5 minutes, 1000 x g at 4°C. The PBS was removed and the cell pellet frozen at -80°C until required.

2.7 Biochemical assays and other methods of analysis

2.7.1 Cell membrane preparation

Cell pellets from transfected cells were thawed and resuspended in 1mL of TE buffer. The cells were homogenised using 40 strokes of a glass on Teflon homogeniser. The cells were centrifuged at 1000 x g for 10 minutes in order to separate any unbroken cells and nuclei at 4°C. The supernatant fraction was removed and passed through a 25 gauge needle 10 times before being transferred to ultra-centrifuge tubes and subjected to centrifugation at 218000 x g for 30 minutes using a Beckman Optima TLX Ultracentrifuge (Palo Alto, CA). The supernatant was discarded and the pellet resuspended in 200µL of TE buffer using a 25 gauge needle, passing through 10 times to ensure an even suspension. The protein concentration was assessed using a BSA assay and membranes diluted to 1µg/µL and

stored at -80°C until required.

2.7.2 BCA protein quantification

Protein amount in membrane preparations was assessed using a BCA assay. This assay utilises bincinhoninic acid (BCA) and copper sulphate solutions in which proteins reduce the Cu(II) ions to Cu(I) ions in correlation with protein amount initiating a colour change caused by BCA binding reduced Cu(I) . This coloured solution has an absorption maximum of 562nm, allowing quantitation of the protein amount. A standard curve was established using standard BSA solutions allowing the concentrations of unknown samples to be established. Solutions used consisted of; reagent A – 1% (w/v) BCA, 2% (w/c) Na_2CO_3 , 0.16%(w/v) sodium tartrate, 0.4% NaOH , 0.95% NaHCO_3 , pH 11.25 and reagent B – 4% CuSO_4 . 1 part reagent B was mixed with 49 parts reagent A and 200 μL of this solution added to 10 μL of protein standard or unknown sample in a 96 well ELISA plate. The assay was incubated at 37°C for 25 minutes before reading the absorbance.

2.7.3 Competition radioligand binding

Reaction mixtures were established in a volume of 100 μL containing 5 μg of protein, 50pM ^3H -IL-8, and a range of competing ‘cold’ ligand concentrations. Samples were incubated for 90 minutes at room temperature prior to vacuum filtration through GF/C filters. Data were analysed using GraphPad Prism, plotted as % specific radioligand binding against ‘cold’ ligand concentration and an EC_{50} value determined via non-linear regression using a one-site competition analysis. The equilibrium dissociation constant for the binding of the

competing 'cold' drug (K_i) was calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) using the homologous competition binding without ligand depletion equation. Results for three independent experiments were analysed using one way ANOVA statistical analysis and Dunnett's post test comparing results obtained for wild type receptor to results from modified constructs.

Ligand depletion can occur in experiments where a large proportion of the radioligand is bound to the receptors. This results in differing concentrations of free ligand existing between assay tubes despite adding the same amount of radiolabelled ligand. When performing a competition assay, high concentrations of the cold competing drug can compete with the labelled ligand for receptor binding and thus can increase the free concentration of the labelled ligand. Non-specific binding values can also be affected by ligand depletion as non-specific binding is directly proportional to the free concentration of labelled ligand and therefore can vary tube to tube. Ligand depletion was observed in these experiments as described in chapter three.

2.7.4 cAMP determination

Second messenger activation was assessed using the HitHunterTM cAMP XS assay kit. This assay utilises enzyme fragment complementation technology (EFC) whereby *E. coli* β -galactosidase is split into two fragments – a large fragment termed enzyme acceptor and a small protein fragment termed enzyme donor. These fragments are inactive when present individually but when combined they rapidly recombine to form an active enzyme. cAMP produced from the cells competes for antibody binding against cAMP conjugated to the

enzyme donor protein fragment. Any unbound ED-cAMP can complement EA forming an active enzyme detected by luminescence. Transfected cells were harvested and the cell pellet resuspended in 1xPBS containing 0.5mM IBMX. A volume corresponding to 20000 cells/well was added to a white-walled 96-well plate (PerkinElmer) and the assay performed in accordance with manufacturers instructions. cAMP was generated using 10 μ M forskolin and agonist applied to the cells acted on adenylyl cyclase to inhibit cAMP. Luminescence was measured using a Mithras LB 940 (Berthold Technology, Bad Wildbad, Germany).

2.7.5 Co-immunoprecipitation

Cells were harvested 24 hours following transfection and resuspended in 2.5mL of 1X RIPA buffer supplemented with 10mM NaF, 5mM EDTA pH 8, 10mM NaH₂PO₄, 5% ethylene glycol and a Complete EDTA-free protease inhibitor tablet. The cell pellet was resuspended using a pipette and placed on a rotating wheel at 4°C for 1 hour. The samples were then centrifuged for 10 minutes at 20817 x g at 4°C and the supernatant transferred to a fresh tube containing 200 μ L of 1X RIPA and 50 μ L of Protein G to pre-clear the samples. This was incubated at 4°C on a rotating wheel for 1 hour. The samples were then subjected to centrifugation at 20817 x g at 4°C for 1 min and the supernatant transferred to a fresh tube and the protein concentration determined. The protein concentration was equalised to 1 μ g/ μ L using 1 X RIPA and 600 μ L of each sample was incubated overnight with 40 μ L Protein G, 5 μ g M2 anti-Flag antibody at 4°C on a rotating wheel. 100 μ L of the equalised supernatant was reserved in order to investigate protein expression in the cell lysates.

Approximately 16 hours following incubation, the samples were centrifuged at $20817 \times g$ for 1 min at 4°C and the Protein G beads washed in 1xRIPA buffer. This was repeated a further twice before adding 40 μl Laemmli buffer and the samples heated to 85°C for 4 minutes to elute the protein. Both immunoprecipitated samples and cell lysates were then subjected to SDS-PAGE gel electrophoresis.

2.7.6 SDS-PAGE and western blotting

Protein samples were resolved using SDS-PAGE. Precast Novex Bis-tris gels were used at 4-12% acrylamide concentration. Nupage MOPS SDS buffer was used for electrophoresis using the XCell Surelock mini-cell gel tank apparatus. The gels were run at 200V for approximately 45 minutes. The proteins were transferred onto nitrocellulose membrane using the XCell II blot module apparatus. The membrane and components of the transfer apparatus were soaked in transfer buffer (0.2M glycine, 25mM Tris and 20% methanol) before initiating transfer. Proteins were transferred at 30V for approximately 1 hour. Efficient transfer was investigated by staining with Ponceau stain (0.1% (w/v) Ponceau S, 3% (w/v) trichloroacetic acid). In order to block non-specific binding sites, membranes were incubated in 5% (w/v) low fat milk, 0.1% Tween 20/PBS (v/v) solution at room temperature on a rotating shaker for two hours. The membrane was incubated with primary antibody overnight in 5% (w/v) low fat milk, 0.1% Tween 20/PBS (v/v) solution at 4°C . Approximately 16 hours later the membrane was washed 3 times in 0.1% Tween 20/PBS for five minutes each wash. The secondary antibody which was horseradish peroxidase linked was incubated again in 5% (w/v) low fat milk, 0.1% Tween 20/PBS (v/v) solution and incubated for 20 minutes at room temperature. Again the membrane was washed 3 times in 0.1% Tween 20/PBS for five minutes each wash prior to application of ECL

solution and developing of the blot. The membranes were exposed to blue Kodak film and developed using a Xomat machine. For co-immunoprecipitation reactions the blot was stripped and re-probed using different antibodies. The membrane was washed using RcBlot Plus solution diluted 1:10 in H₂O for 15 minutes and then again incubated with 5% (w/v) low fat milk, 0.1% Tween 20/PBS (v/v) solution to block non-specific sites.

The antibody dilutions for western blot analysis were;

<i>Primary Antibody</i>	<i>Dilution Factor</i>	<i>Secondary Antibody</i>
Anti-c-Myc	1:1000	Anti-rabbit
Anti-Flag M2	1:2000	Anti-mouse

The dilution factor for secondary antibody in each case was 1:10000.

2.7.7 BRET²

2.7.7.1 Single point BRET²

Cells were washed twice in 1X PBS supplemented with 1g/L glucose and resuspended in a final volume of 1mL. 160µL of cells were dispensed into a white-walled 96 well plate and either 20µL of agonist added or PBS/glucose. If agonist was tested then the plate was incubated for 30 minutes at 37°C. DeepBlueC substrate was diluted 1:20 in PBS/glucose and the mix kept protected from light until required. 20µL of substrate was added to each well resulting in final concentration of 10µM and BRET² measured using a Mithras LB940 (Berthold Technology, Bad Wildbad, Germany). Readings were taken using a 410nm (band pass 80nm) filter corresponding to light emission resulting from luciferase catalysing the substrate to coelenteramide. Energy transfer emitted by GFP² was detected using a 515nm (band pass 30nm) filter and a ratiometric reading obtained corresponding to the

ratio of light intensity (515nm) to light intensity (410nm).

2.7.7.2 Saturation BRET²

In saturation experiments cells were transfected with a constant amount of *Renilla* Luciferase-linked (Rluc) construct and a varying amount of acceptor GFP² construct. These cells were harvested and cell membranes prepared and diluted to 0.5µg/µL. BRET² was assessed as previously described for intact cells. Luminescence and fluorescence measurements were also obtained. 50µL of cell membranes were dispensed into white-walled 96-well plates (PerkinElmer) for luminescence measurements and black-walled 386-plates (Costar, Cambridge, MA) for fluorescence measurement. For luminescence measurement h-Coelenterazine (Prolume) was added to the cell membranes yielding a final concentration of 5µM and the plate incubated at room temperature for 30 minutes prior to reading at 410nm using the Mithras LB 940 (Berthold Technology, Bad Wildbad, Germany). GFP² fluorescence was assessed using the Victor² 1420 Multilabel counter (PerkinElmer). BRET² readings were corrected for energy transfer resulting from 'bleedthrough' of the Rluc construct expressed alone but detected in the GFP² channel. Fluorescence readings were corrected for the endogenous fluorescence detected for non-transfected HEK293T cell membranes. Fluorescence readings were expressed over luminescence and BRET² ratios plotted forming a saturation curve. GraphPad Prism 4 was used to analyse data using a one site binding hyperbola equation yielding BRET_{MAX} and BRET₅₀ values.

2.7.8 Time resolved FRET

10cm² dishes of HEK293T cells were transfected to express CXCR1 or CXCR2 N-terminally modified to express either c-Myc or Flag epitope tags. 48 hours following transfection the cells were harvested and the samples containing singly tagged receptors mixed to form a negative control. The cell pellets were resuspended in 200μL of ice-cold PBS. Antibodies directed against the epitope tags c-Myc and Flag conjugated to the donor and acceptor moieties Europium³⁺ (Eu³⁺) and Allophycocyanin (APC) were used to investigate energy transfer. The anti-c-Myc Eu³⁺ and anti-Flag APC antibodies were diluted in 50% NCBS: 50% PBS to a final concentration of 5nM and 15nM respectively. A dilution containing the anti-c-Myc Eu³⁺ antibody only was also prepared. 250μL of the antibody dilutions were aliquoted into fresh tubes and 50μL of resuspended cells transferred resulting in antibody incubations containing only anti-c-Myc Eu³⁺ or containing both anti-c-Myc Eu³⁺ and anti-Flag APC antibodies for each sample. The samples were mixed and then incubated on a rotating wheel at room temperature for 2 hours. The samples were covered in aluminium foil to minimise exposure of the fluorophores to light. The samples were then centrifuged at 1000 x g for 1 minute and the antibody mix removed from the cell pellet. The pellet was then washed 2X in ice-cold PBS and resuspended in 250μL of PBS. To investigate agonist effect on energy transfer, 90μL of cells were transferred to a fresh tube and incubated with the chosen concentration of agonist at 37°C. In order to measure the energy transfer, 40μL of each sample was dispensed in triplicate into a black 384-well plate. Blank wells containing PBS were also included.

Tr-FRET was determined using a Victor² plate reader (PackardBioscience). Excitation was at 340nm and emission filters generated data representing donor (615nm) and acceptor (665nm) emission. Normalized FRET was calculated using the equation;

$$\text{Normalized FRET} = ((A_{665} - \text{BLK})/D_{615}) - C$$

Where A_{665} is the fluorescent emission from the acceptor, D_{615} is the fluorescent emission from the donor and BLK represents the background reading at 665nm from the wells containing PBS. C represents the crosstalk between the donor and acceptor windows for the samples incubated with only anti-c-Myc Eu^{3+} and is equal to $A_{665} - \text{BLK}/D_{615}$.

2.7.9 Fluorescent microscopy and FRET imaging in living cells

HEK293T cells were grown on coverslips treated with poly-D-lysine and transiently transfected with the different CFP/YFP fusion proteins. Coverslip fragments were placed into a microscope chamber containing physiological saline solution (130mM NaCl, 5mM KCl, 1mM CaCl_2 , 1mM MgCl_2 , 20mM HEPES, 10mM D-glucose pH 7.4). Cells were visualised using a Nikon Eclipse TE2000-E fluorescence inverted microscope and images obtained individually for YFP, CFP and FRET filter channels using a Optoscan monochromator (Cairn Research, Faversham, Kent, UK) and a dichroic mirror 86002v2bs (Chroma Inc., Rockingham, VT). The filter sets used were; eYFP (excitation – 500/5nm; emission – 535/30nm), CFP (excitation 430/12nm; emission – 470/30nm) and FRET (excitation – 430/12nm; emission – 535/30nm). The illumination time was 250ms and binning modes 2x2. MetaMorph imaging software (Universal Imaging Corp., West Chester, PA) was used to quantify the FRET images using the sensitised FRET method. Corrected FRET was calculated using a pixel-by-pixel methodology using the equation $\text{FRETc} = \text{FRET} - (\text{coefficient B} \times \text{CFP}) - (\text{coefficient A} \times \text{eYFP})$, where CFP, eYFP and FRET values correspond to background corrected images obtained through the CFP, eYFP and FRET channels. B and A correspond to the values obtained for the CFP (donor) and

eYFP (acceptor) bleedthrough coefficients respectively, calculated using cells singly transfected with either the CFP or YFP protein alone. The values obtained for the constructs used in this study were;

<i>Construct</i>	<i>Coefficient</i>
CXCR1YFP	0.197
CXCR1CFP	0.662
CXCR2YFP	0.197
CXCR2CFP	0.660
α_{1A} -YFP	0.196
DOP-YFP	0.197
MOP-YFP	0.192
KOP-YFP	0.200

To correct the FRET levels for the varying amounts of donor (CFP) and acceptor (eYFP), normalised FRET was calculated using the equation $FRET_n = FRET_c / CFP \times eYFP$, where $FRET_c$, CFP and eYFP are equal to the fluorescence values measured for each

individual cell. FRET was measured for 6-8 cells per experiment with at least three individual transfections performed.

2.7.10 Confocal laser scanning microscopy

Cells were imaged using a laser scanning confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss Inc., Thornwood, NY) equipped with a 63x oil-immersion Plan Fluor Apochromat objective lens with a numerical aperture of 1.4. A pinhole of 20 and an electronic zoom of 1 or 2.5 was used. The excitation laser line for GFP and YFP was 488nm argon laser and detected with a 505-530 band pass filter. Alexa 594 label was detected using a 543nm helium/neon laser and detected with a 560 long-pass filter. The images were manipulated using MetaMorph imaging software (Version 6.1.3; Universal Imaging Corporation, Downing, PA, USA).

2.7.10.1 Fixed cell samples

Cells grown on coverslips were transiently transfected and washed three times with ice-cold PBS. Cells were fixed for 10 minutes at room temperature using 4% paraformaldehyde in PBS/5% sucrose solution. The cells were washed a further three times in ice-cold PBS prior to being fixed onto microscope slides with 40% glycerol in PBS.

2.7.10.2 Immunostaining protocol

Immunostaining was performed to monitor agonist-induced internalisation of the N-terminal tagged receptors. Cells were grown onto coverslips and transiently transfected. 24

hours following transfection the medium was removed and the cells incubated with 20mM HEPES/DMEM containing the appropriate dilution of primary antibody and incubated for 40 minutes at 37°C in 5% CO₂. Either IL-8 or GRO- α was added to yield a final concentration of 50nM and the coverslip incubated for a further 30 minutes at 37°C, 5% CO₂. Coverslips were washed three times with PBS and cells fixed by incubating with 4% paraformaldehyde in PBS/5% sucrose solution for 10 minutes at room temperature. The coverslips were washed a further three times in PBS prior to being permeabilised in 0.15% Triton-X-100/3% low fat milk/PBS for 10 minutes at room temperature. The coverslips were incubated with a secondary antibody conjugated to an Alexa 488/594 fluorophore at a dilution of 1:400 corresponding to an antibody concentration of 5 μ g/ μ L. This incubation was performed with the coverslip cell side down onto Nescofilm, for 1 hour at room temperature. Following incubation the cells were washed twice in 0.15% Triton-X-100/3% low fat milk/PBS and three times in PBS. Coverslips were then mounted onto microscope slides with 40% glycerol in PBS. The antibody dilutions were;

<i>Primary antibody</i>	<i>Dilution factor</i>	<i>Secondary antibody</i>
Anti-c-Myc	1:100	Anti-rabbit
Anti-Flag	1:100	Anti-mouse
Anti-HA	1:2000	Anti-mouse
Anti-VSV	1:2500	Anti-mouse
Anti-CXCR1	1:50	Anti-mouse
Anti-CXCR2	1:50	Anti-mouse

2.7.11 Retention motif - cell surface expression quantification

Cells were co-transfected with Flag-CXCR1 and either HA-CXCR1 or IIA-CXCR1-ER. 48 hours following transfection cells were harvested and counted using a haemocytometer. Approximately 500 000 cells were dispensed into a tube and the cells incubated with 15nM anti-Flag APC antibody and 1 μ M Hoechst stain diluted in 50% NBBS: 50% PBS on a rotating wheel for 1 hour. The cells were centrifuged at 1000 x g for 1 minute and the cell pellet washed three times in PBS. The cells were resuspended in 200 μ l of PBS and 40 μ l replicates dispensed into a black 384-well plate. The fluorescence was quantified using the Victor² 1420 Multilabel counter (PerkinElmer). A control in which fluorescence was measured in non-transfected HEK293T cells was performed and this value removed from the other readings, representing endogenous fluorescence of the cell type. As a further control to ensure equal cell number between wells, fluorescence corresponding to Hoechst staining was measured and the data analysed to ensure cell number did not exceed 20% variance from well to well.

Chapter 3

3.1 Introduction

In order to utilise currently available biochemical and biophysical techniques to investigate oligomerisation, several different N- and C- terminal epitope tags and fluorescent moieties were introduced to both CXCR1 and CXCR2.

N-terminal epitope tags have been used extensively as tools in biochemical techniques facilitating protein detection without the requirement for receptor specific antibodies. Antibodies to several commonly used epitope tags are commercially available and can be used for several purposes including subcellular protein localisation and investigation of protein-protein interactions. In this study N-terminal Flag, c-Myc, HA and VSV epitope tags have been used, located immediately following the initiating methionine. Several of the currently available epitope tags are derived from naturally occurring proteins. The c-Myc epitope is a decapeptide sequence originating from the human c-Myc protein, found in several types of human tumours (Evan *et al.*, 1985). The HA epitope originates from the hemagglutinin protein which is a surface glycoprotein required for the infectivity of the human influenza virus (Wilson *et al.*, 1984). The VSV epitope tag is an eleven amino acid sequence derived from the vesicular stomatitis virus (VSV) glycoprotein, a virally encoded transmembrane protein (Kreis, 1986). The FLAG tag is a synthetic octapeptide (Chubet and Brizzard, 1996).

Green fluorescent protein (GFP) from the jellyfish *Aequoria Victoria* is a commonly used reporter molecule permitting the detection of protein expression and localisation within the

cell. GFP is an auto-fluorescent protein of 27kDa that can be located either at the N- or C-terminal of the protein of interest. Several variants of GFP have been isolated, possessing distinct spectral properties that have been exploited in several resonance energy transfer techniques. GFP² is a modified form of GFP that has been developed by PerkinElmer to maximally absorb the energy released by the oxidation of the substrate DeepBlueC by *Renilla* luciferase. Oxidation of DeepBlueC to coelenteramide results in the release of energy at 395nm overlapping with the excitation spectrum of GFP² and resulting in subsequent emission at 510nm. Enhanced cyan fluorescent protein (eCFP) is a variant of GFP that has a blue-green colour (Cubitt *et al.*, 1999; Heim and Tsien, 1996). It has an excitation maximum of 430nm and emission maximum 470nm. A red-shifted GFP variant termed enhanced yellow fluorescent protein (eYFP) presents an excitation maximum of 500nm and emission maximum of 535nm. The spectral properties of eCFP and eYFP have been extensively exploited as donor and acceptor moieties in FRET to demonstrate molecular interactions between proteins that are both cytoplasmic and membrane associated, including GPCRs (Tsien, 1998; Zhou *et al.*, 2003; Dinger *et al.*, 2003). In this study the fluorescent moieties have been ligated onto the C-terminal of the chemokine receptors.

As mentioned previously CXCR1 and CXCR2 bind their ligands via an interaction with the N-terminal region and the third extracellular loop of the receptor (LaRosa *et al.*, 1992). Therefore it is important to ensure that the addition of N-terminal epitope tags onto these receptors does not interfere with the ability of the receptors to bind ligand or the affinity of this ligand interaction. The addition of C-terminal fluorescent moieties could potentially interfere with the ability of the receptor to interact with the endocytic machinery responsible for receptor sequestration and/or influence G protein coupling.

The aim in this chapter was to investigate the ability of modified CXCR1 and CXCR2 constructs to display native pharmacology. The ability of the modified receptors to undergo ligand-induced internalisation was investigated using confocal microscopy. Radioligand binding was used to ensure the ligand binding profiles of the modified receptors did not differ substantially from wild type receptor. The ability of the modified receptors to couple to G proteins was assessed by investigating the reduction in forskolin-stimulated cAMP release, caused by interaction of CXCR1 and CXCR2 with $G\alpha_i$. (Wu *et al.*, 1993; Damaj *et al.*, 1996).

3.2 Internalisation of modified forms of CXCR1 and CXCR2

The ability of the modified constructs to undergo agonist-induced internalisation was assessed by immunocytochemical staining of N-terminal epitope tags and direct visualisation of C-terminal fluorescent moieties using confocal microscopy. HEK293T cells were transiently transfected with receptor cDNA 24 hours prior to visualisation. Figure 3.1 demonstrates that, prior to exposure to agonist, the modified CXCR1 constructs were clearly plasma membrane delineated. CXCR1GFP², CXCR1YFP and CXCR1CFP presented some cytoplasmic retention, however the majority of these receptors were expressed at the plasma membrane. Following treatment with 50nM IL-8 for 30 minutes at 37°C, a clear punctate pattern corresponding to a loss of cell surface expression could be observed for all constructs and representing the internalisation of the receptor into intracellular vesicles. Although no quantitative measurements were made, the internalisation of the CXCR1-fluorescent protein conjugates appeared to be to a lesser extent than observed for N-terminally epitope tagged receptor forms, with a proportion of

the receptor conjugate remaining membrane delineated following agonist exposure.

Figure 3.2 illustrates the expression patterns of the modified CXCR2 constructs. In the absence of ligand the receptors were clearly plasma membrane localised. Some cytoplasmic retention of the CXCR2GFP², CXCR2CFP and CXCR2YFP constructs was observed, but as with CXCR1GFP² the majority of the receptors were plasma membrane delineated. Following incubation with 50nM IL-8 for 30 minutes at 37°C, the modified forms of CXCR2 underwent pronounced internalisation demonstrated by the absence of membrane localisation and the appearance of punctate intracellular vesicles. As was observed for CXCR1, the extent of internalisation of CXCR2-fluorescent protein conjugates was less than observed for N-terminally tagged CXCR2 constructs.

Figure 3.3 demonstrates the plasma membrane expression pattern of CXCR1Rluc and CXCR2Rluc. These receptors were visualised using antibodies specific to the N-terminal regions of CXCR1 and CXCR2 that prevent ligand binding. Therefore the ability of these receptors to undergo agonist-induced internalisation could not be investigated.

3.3 [¹²⁵I]IL-8 binding to modified forms of CXCR1 and CXCR2

Competition binding experiments were performed for both native and modified forms of CXCR1 and CXCR2 in order to ensure that the affinities of the recombinant receptors for IL-8 were similar to that of native receptor protein. HEK293T cells were transiently transfected with the various forms of CXCR1 cDNA and harvested 48 hours following transfection. Membranes were prepared and used in competition binding experiments, the results of which are detailed in Figure 3.4. Unlabelled IL-8 fully competed for the binding

of native CXCR1 with radiolabelled IL-8 yielding a pK_i value of 9.5 ± 0.3 M. Homologous competition binding experiments performed investigating IL-8 affinity on modified forms of CXCR1 yielded pK_i values that did not vary significantly from that obtained for native receptor when assessed by one way ANOVA statistical analysis using a Dunnett post test. Ligand depletion was calculated in each reaction. Unfortunately due to the experimental conditions used ligand depletion at levels greater than 10% was observed as detailed in Table 3.1. However, when the data was examined using the GraphPad prism calculation homologous competition binding with ligand depletion. This demonstrated that the pK_i values recorded did not vary significantly.

Similar experiments were performed on the modified forms of CXCR2. The native CXCR2 yielded a pK_i value of 9.51 ± 0.1 M (Figure 3.5). Statistical analysis performed on pK_i values obtained for the modified forms of CXCR2 did not highlight any significant variation in affinity from native CXCR2.

3.4 IL-8 induced inhibition of forskolin-stimulated cAMP release

Cyclic AMP levels were measured using the DiscoverX cAMP XS kit optimised to detect $G\alpha_i$ activation. Cells transiently expressing CXCR1 were incubated with $10\mu\text{M}$ forskolin and various levels of IL-8 for 30 minutes and cAMP levels recorded (Figure 3.6). The concentration of IL-8 causing maximal inhibition of forskolin-induced cAMP production was used to investigate the ability of modified forms of CXCR1 to interact with G protein. Figure 3.7 demonstrates the ability of 100nM IL-8 to inhibit forskolin stimulated cAMP release. In all cases IL-8 caused some degree of inhibition of forskolin-induced cAMP generation. However, statistical analysis demonstrates that the inhibition of forskolin-

stimulated cAMP was not significantly different from basal values for every receptor examined.

Similar studies were performed for the modified forms of CXCR2. A dose-inhibition curve demonstrating agonist-induced inhibition of forskolin-induced cAMP generation for CXCR2 is shown in Figure 3.6. Again the concentration of IL-8 yielding maximal inhibition was selected and used to investigate G protein activation for the modified forms of CXCR2 (Figure 3.8). This demonstrated the ability of all the modified forms of CXCR2 to attenuate forskolin-induced cAMP production via interaction with G protein. As observed for CXCR1, variations in degree of inhibition were observed and statistical tests demonstrated that the extent of forskolin-induced cAMP generation was not significantly different from basal for some receptors examined.

3.5 Discussion

The introduction of N- and C-terminal modifications to GPCRs could potentially have a large impact on receptor function. The N-terminal region of chemokine receptors has been implicated in ligand binding and the modification of this region could influence the capacity of receptors to bind ligand and also affect ligand binding affinity. The C-terminal region of GPCRs is frequently very important in regulating receptor endocytosis and G protein interactions. A single amino acid change in the C-terminal tail of the β_2 -adrenergic receptor was sufficient to disrupt interactions with intracellular endocytic machinery and influence GPCR trafficking (Hall *et al.*, 1998; Cao *et al.*, 1999). The addition of large fluorescent moieties to the C-terminal region of GPCRs could therefore potentially abrogate receptor endocytosis.

Experiments performed in this study investigating the internalisation properties of the modified forms of CXCR1 and CXCR2 demonstrated the ability of the N- and C-terminally modified receptors to undergo pronounced receptor endocytosis following exposure to agonist. However, CXCR1 and CXCR2 receptors conjugated to fluorescent C-terminal moieties demonstrated comparatively higher levels of residual plasma membrane expression following exposure to agonist than observed for N-terminally modified constructs. Studies performed using GPCR-GFP fusions have reported markedly slower internalisation kinetics in comparison to the wild type GPCR (McLean and Milligan, 2000). This could explain the residual membrane delineation observed for GPCR-fluorescent protein conjugates in this study.

Some intracellular retention of C-terminally modified forms of CXCR1 and CXCR2 was observed. Retention of CXCR1GFP within the Golgi apparatus was also observed by Barlic *et al.*, (1999) following transient expression in unstimulated HEK293 cells. This could be due to the long half-life of the fluorescent moieties used in this study resulting in the maintained presence of the receptor-fluorescent protein fusion within intracellular vesicles (McLean and Milligan, 2000). However, it is possible that expression levels resulting from the transient expression of these constructs are at a level sufficient to overwhelm N-terminal glycosylation capacity, resulting in intracellular accumulation of the receptor.

The cell line used in these experiments was the HEK293T line. Barlic *et al.*, (1999) investigated internalisation of CXCR1GFP in HEK293 cells. However, receptor internalisation could not be observed in this cell type unless the receptor was co-

transfected with β -arrestins and GRK2, thought to be due to low endogenous levels of these proteins in the cell line used. Internalisation of the modified forms of CXCR1 and CXCR2 in this study did not require the enhanced expression of these proteins, suggesting endogenous expression levels in the HEK293T cell line used were sufficient for receptor endocytosis. A study performed by Rose *et al.*, (2004) compared the internalisation kinetics of CXCR1 and CXCR2 expressed both endogenously in neutrophils and in transiently transfected HEK293 cells. The authors reported very similar internalisation kinetics between neutrophils and HEK293 cells, however the recycling properties of the two receptors differed markedly between the two cell types.

The addition of N- or C- terminal tags to CXCR1 and CXCR2 did not alter the affinity of these receptors for IL-8 which yielded pK_i values of ~ 9.5 M for all constructs tested. The K_i values determined in this study are consistent with binding affinities determined in both cell types that endogenously express these receptors and recombinant systems (Lee *et al.*, 1992; Loetscher *et al.*, 1994; Ahuja and Murphy, 1996; Wu *et al.*, 1996). Binding affinities of receptor-fluorescent protein conjugates have been investigated for several receptors with no change in reported ligand binding characteristics. This has been demonstrated for several GPCRs including the CCK₁ receptor (Tarasova *et al.*, 1997), β_1 adrenoceptor (Mercier *et al.*, 2002), β_2 adrenoceptor (Kallai *et al.*, 1998; Barak *et al.*, 1997), TRH1 receptor (Drnotta *et al.*, 1998), α_{1A} and α_{1B} adrenoceptors (Hirasawa *et al.*, 1997; Stanasila *et al.*, 2002), vasopressin V₂ receptor (Schulein *et al.*, 1998), melatonin MT₁ and MT₂ receptors (Ayoub *et al.*, 2002) and the GnRH receptor (Nelson *et al.*, 1999). The addition of N-terminal epitope tags has also been shown to have no effect on ligand binding. This has been shown for several GPCRs including the α_{1A} adrenoceptor (Stanasila *et al.*, 2002) and vasopressin V_{1a} and V₂ vasopressin receptors (Terrillon *et al.*, 2004).

In this study, G protein coupling was assessed by investigating agonist-induced reduction in forskolin-stimulated cAMP levels. The assay system used in these experiments was the DiscoverX cAMP assay. This kit did not yield a high signal:noise ratio and several difficulties were experienced while trying to optimise the assay. This is reflected in the data shown in figures 3.7 and 3.8 in which the reduction in forskolin-induced cAMP production has been found not to differ significantly from basal values. Therefore, conclusions concerning the ability of the modified receptors to activate G proteins cannot be drawn based on this data. The ability of GPCR-fluorescent protein conjugates to interact with G proteins has been investigated for several other GPCRs including the CCK₁ receptor (Tarasova *et al.*, 1997), β_1 adrenoceptor (Mercier *et al.*, 2002), β_2 adrenoceptor (Kallal *et al.*, 1998, Mercier 2002), TRH₁ receptor (Drnosta *et al.*, 1998), α_{1B} adrenoceptor (Awaji *et al.*, 1998), NK₁ receptor (McConalogue *et al.*, 1999), α_{1A} adrenoceptor (Stanasila *et al.*, 2003), adenosine A_{2A} (Canals *et al.*, 2004) and melatonin MT₁ and MT₂ receptors (Ayoub *et al.*, 2002). The addition of epitope tags has also been shown to have no effect on interaction with G proteins for several receptors including the α_{1A} adrenoceptor (Stanasila *et al.*, 2003).

Several studies have investigated the pharmacology of N and C terminal tagged CXCR1 and CXCR2. Barlic *et al.*, (1999) demonstrated the ability of CXCR1GFP to induce the release of granule contents following agonist exposure, demonstrating that the modified receptors retain the ability to transduce signals. An extensive study performed by Hall *et al.*, (1999) investigated the pharmacology of N-terminal epitope tagged forms of CXCR1 and CXCR2. Several points within the GPCR signalling cascade were investigated including ligand binding affinities, [³⁵S] GTP γ S activation and inhibition of adenylyl

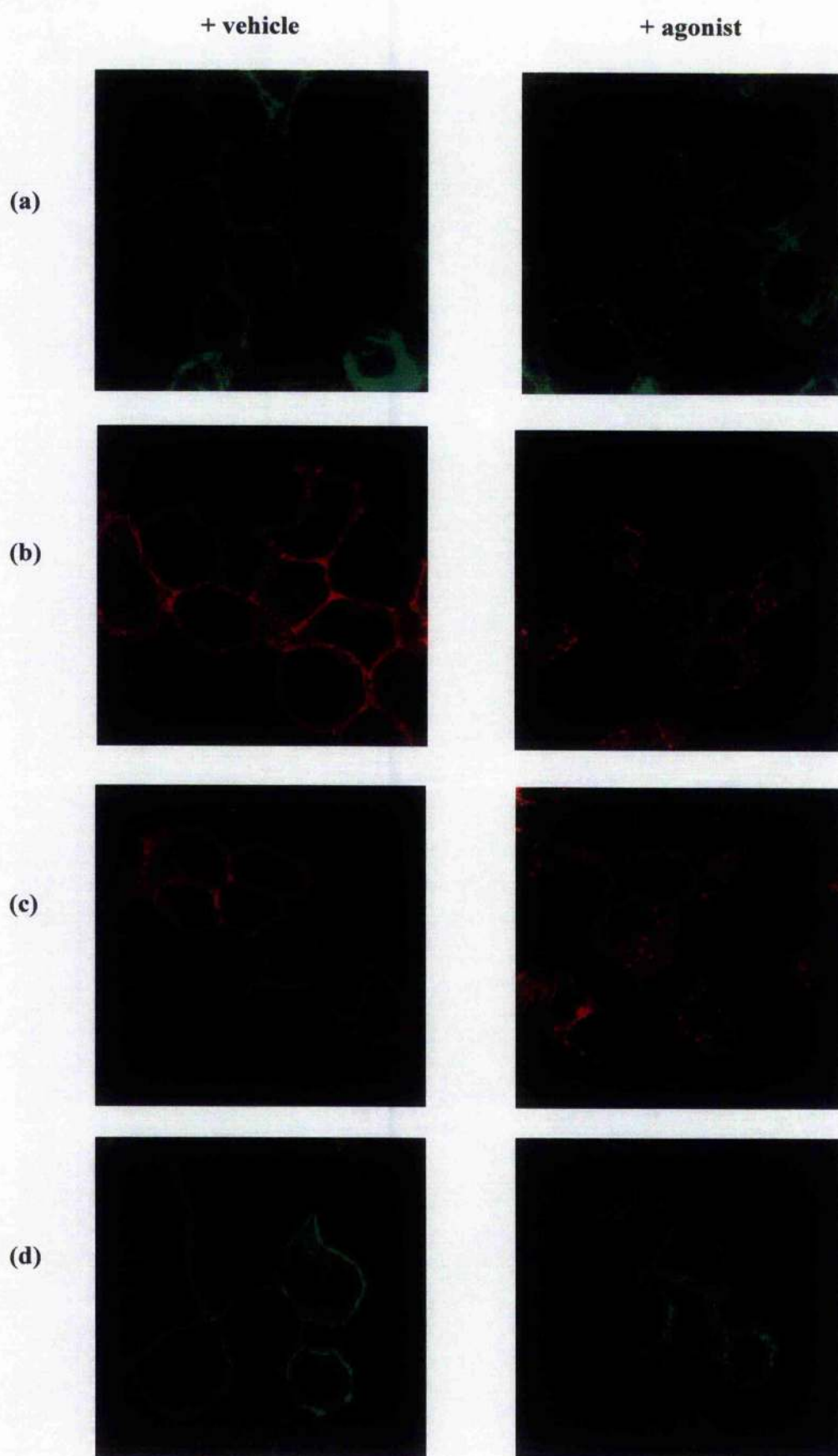
cyclase and demonstrated native receptor pharmacology for the modified receptors.

The data shown in this chapter confirm that the N- and C- terminal modifications introduced to CXCR1 and CXCR2 did not substantially influence the ability of these receptors to bind ligand, undergo receptor endocytosis or couple to G protein.

Figure 3.1 Agonist-induced internalisation of N- and C- terminally modified forms of CXCR1

HEK293T cells were transiently transfected with (a) CXCR1GFP² (b) c-Myc CXCR1 (c) Flag-CXCR1 (d) HA-CXCR1 (e) CXCR1-eYFP or (f) CXCR1CFP. Cells were challenged with vehicle or 50nM IL-8 for 30 minutes prior to fixing. Cells were imaged using confocal microscopy by either immunostaining (b, c, d) or direct visualisation (a, e, f). Results shown are of a single experiment, representative of three experiments performed.

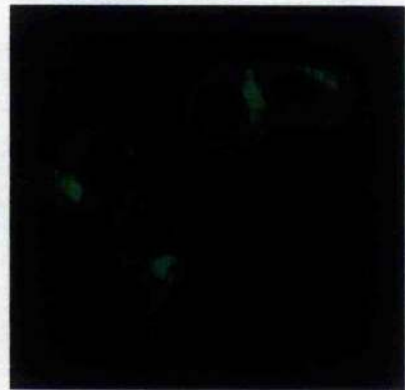
Figure 3.1



+ vehicle

+ agonist

(e)



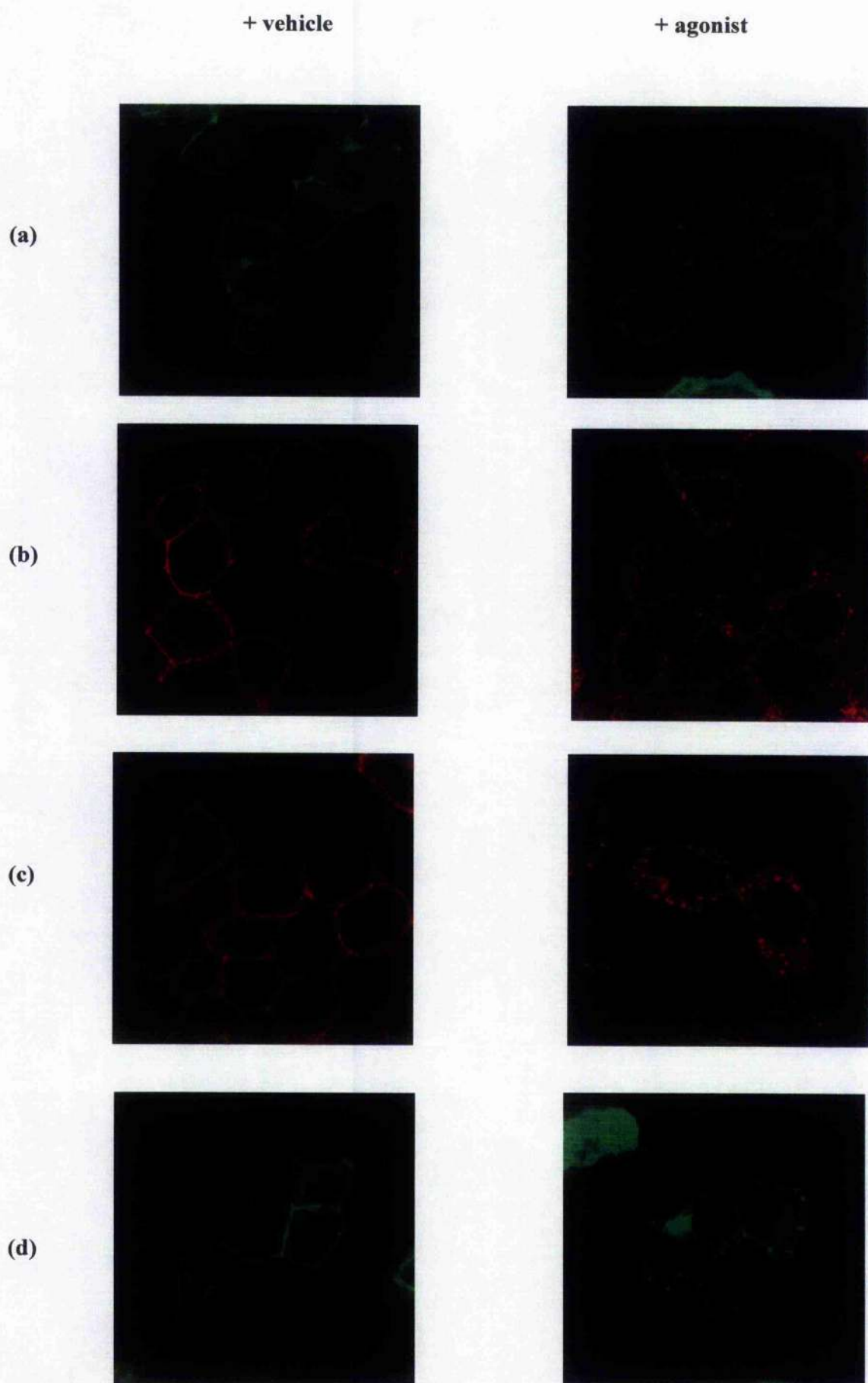
(f)



Figure 3.2 Agonist-induced internalisation of N- and C- terminally modified forms of CXCR2

HEK293T cells were transiently transfected with (a) CXCR2GFP² (b) c-Myc-CXCR2 (c) Flag-CXCR2 (d) CXCR2YFP (e) VSV-CXCR2 or (f) CXCR2CFP. Cells were challenged with vehicle or 50nM IL-8 for 30 minutes prior to fixing. Cells were imaged using confocal microscopy by either immunostaining (b, c, e) or direct visualisation (a, d, f). Results shown are of a single experiment, representative of three experiments performed.

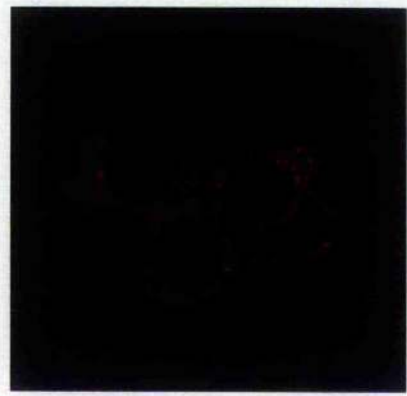
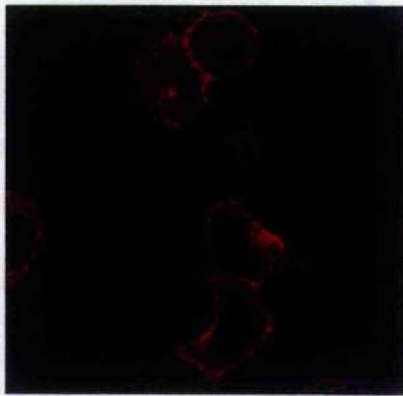
Figure 3.2



+ vehicle

+ agonist

(e)



(f)

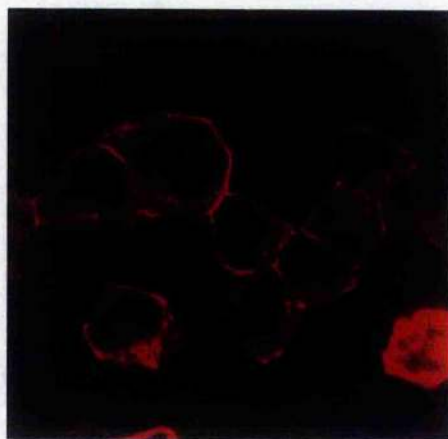


Figure 3.3 Cell membrane expression of CXCR1Rluc and CXCR2Rluc

HEK293T cells were transiently transfected with CXCR1Rluc (a) or CXCR2Rluc (b). Cells were fixed and immunostained with antibodies specific for either CXCR1 or CXCR2 respectively and visualised using confocal microscopy. Results shown are of a single experiment, representative of three experiments performed.

Figure 3.3

(a)



(b)

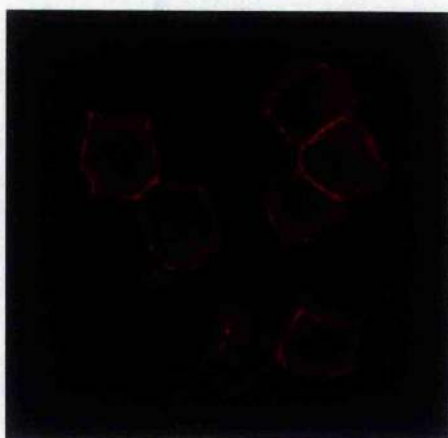
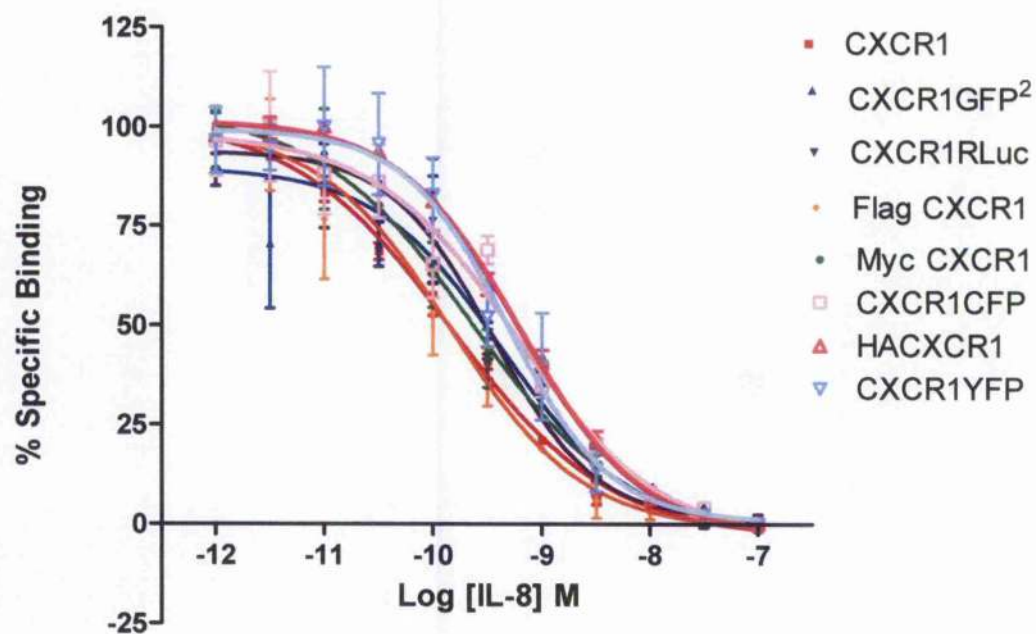


Figure 3.4 N- and C- terminal modifications of CXCR1 have little effect on ligand affinity

Homologous competition binding experiments were performed using [I^{125}] IL-8 and IL-8 for each modified form of CXCR1. Data represent mean \pm S.E.M of three individual experiments. Statistical analysis of results was performed using a one-way ANOVA and Dunnett post test (GraphPad Prism) comparing results from modified receptors to that determined for unmodified CXCR1, demonstrating no significant variance.

Figure 3.4

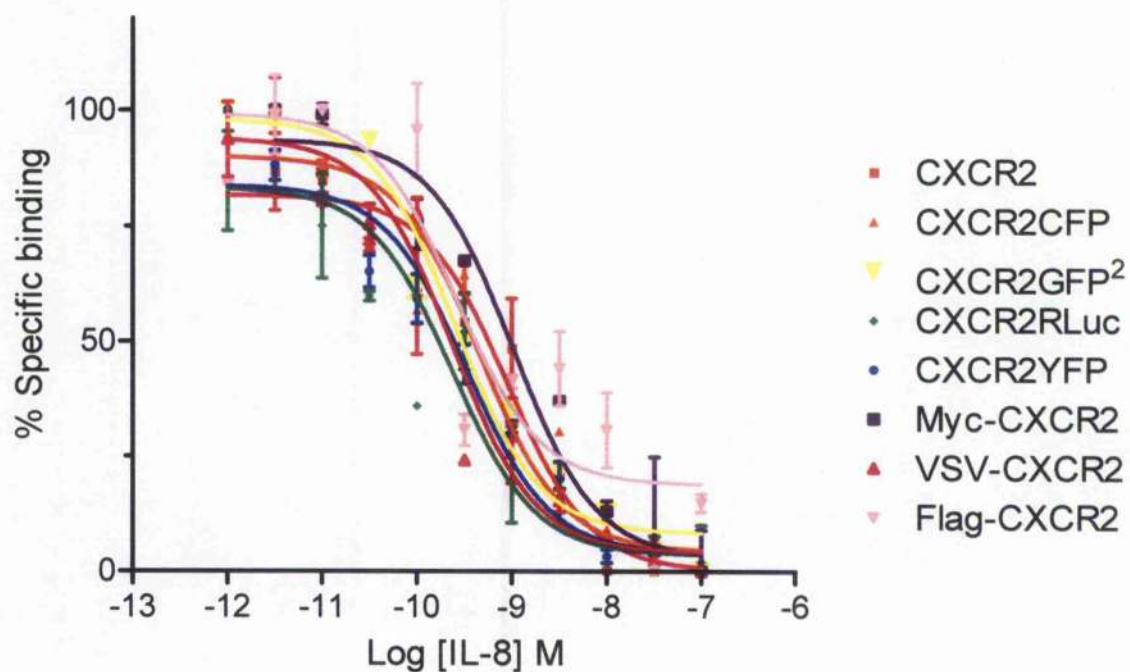


Construct	pKi Value
CXCR1	9.5±0.3
CXCR1GFP ²	9.4±0.2
CXCR1RLuc	9.1±0.1
Flag-CXCR1	9.6±0.1
c-Myc-CXCR1	9.7±0.1
CXCR1YFP	9.4±0.1
CXCR1CFP	9.5±0.2
HA-CXCR1	9.2±0.1

Figure 3.5 N- and C- terminal modifications of CXCR2 have little effect on ligand affinity

Homologous competition binding experiments were performed using [I^{125}] IL-8 and IL-8 for each modified form of CXCR2. Data represent mean \pm S.E.M of three individual experiments. Statistical analysis of results was performed using a one-way ANOVA and Dunnett post test (GraphPad Prism) comparing results from modified receptors to that determined for unmodified CXCR2 demonstrating no significant variance.

Figure 3.5



Construct	pKi Value
CXCR2	9.51±0.1
CXCR2GFP ²	9.5±0.1
CXCR2RLuc	9.5±0.1
Flag-CXCR2	9.5±0.1
c-Myc-CXCR2	9.5±0.5
CXCR2YFP	9.7±0.1
CXCR2CFP	9.4±0.1
VSV-CXCR2	9.6±0.1

Table 3.1 Ligand depletion figures.

The percentage of maximum bound radioligand over actual radioligand added to the assay was calculated for each construct tested. Results are shown as mean \pm S.E.M.

Table 3.1

Construct	Ligand Depletion (%)
CXCR1	20.0±1.6
CXCR1GFP ²	25.1±5.1
CXCR1RLuc	8.4±1.7
Flag-CXCR1	12.3±0.6
c-Myc-CXCR1	21.9±7.9
CXCR1YFP	18.9±1.7
CXCR1CFP	19.0±3.3
HA-CXCR1	26.9±0.6
CXCR2	4.3±0.8
CXCR2GFP ²	4.9±0.9
CXCR2RLuc	9.6±1.7
Flag-CXCR2	5.1±0.9
c-Myc-CXCR2	6.3±3.8
CXCR2YFP	13.3±2.6
CXCR2CFP	21.0±3.3
VSV-CXCR2	6.6±0.6

Figure 3.6 Agonist-induced inhibition of forskolin-induced cAMP generation by CXCR1 and CXCR2

Appropriately transfected cells were stimulated with 10 μ M forskolin and exposed to varying concentrations of IL-8 for 30 minutes prior to cAMP detection using the DiscoverX XS cAMP kit. Data were analysed using the sigmoidal dose-response equation in GraphPad Prism 4. Data shown are representative of three individual experiments.

Figure 3.6

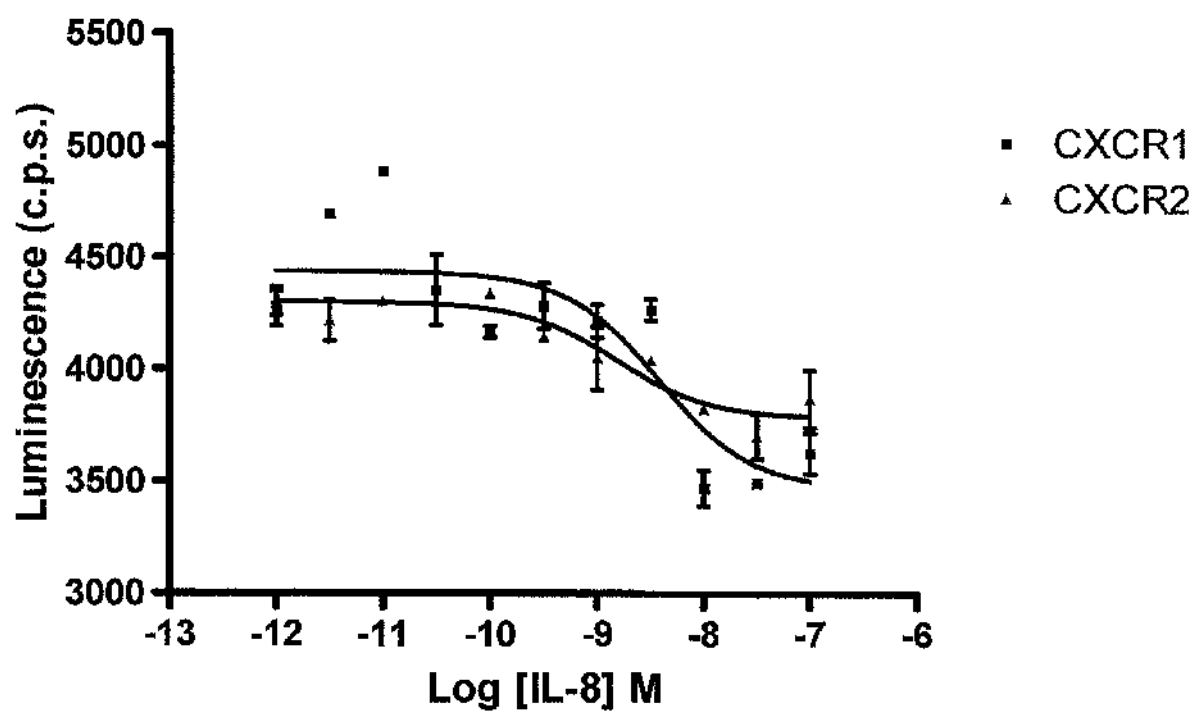


Figure 3.7 Agonist-induced inhibition of forskolin-induced cAMP generation by modified forms of CXCR1

Appropriately transfected cells were stimulated with 10 μ M forskolin and exposed to 100nM IL-8 for 30 minutes prior to cAMP detection using the DiscoverX XS cAMP kit. Data are expressed as percentage of forskolin induced cAMP release. Open bars represent cAMP released following exposure to forskolin (basal) while closed bars represent cAMP levels detected following exposure to IL-8 (+ agonist). Data shown are mean \pm S.E.M and representative of three individual experiments.

Figure 3.7

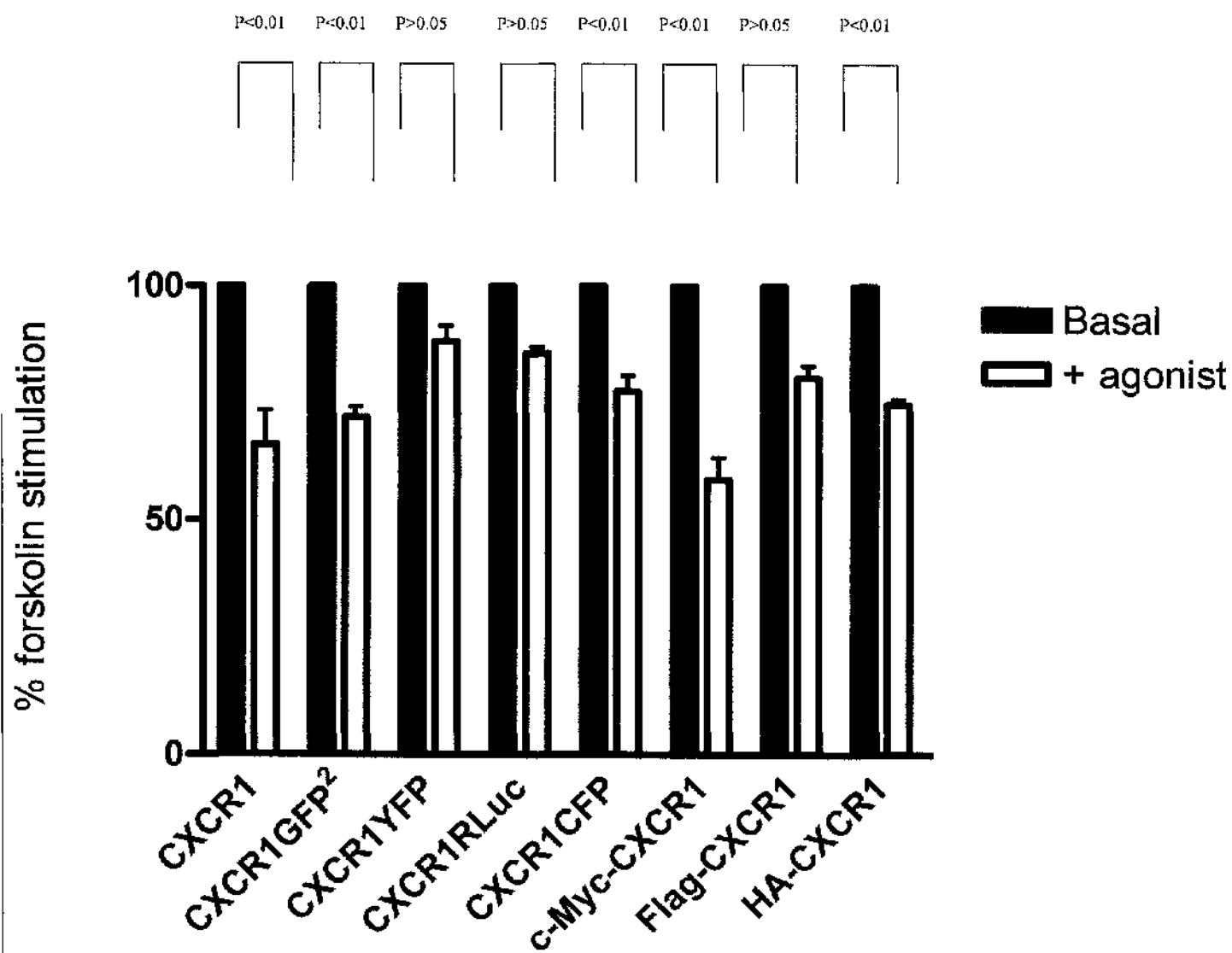
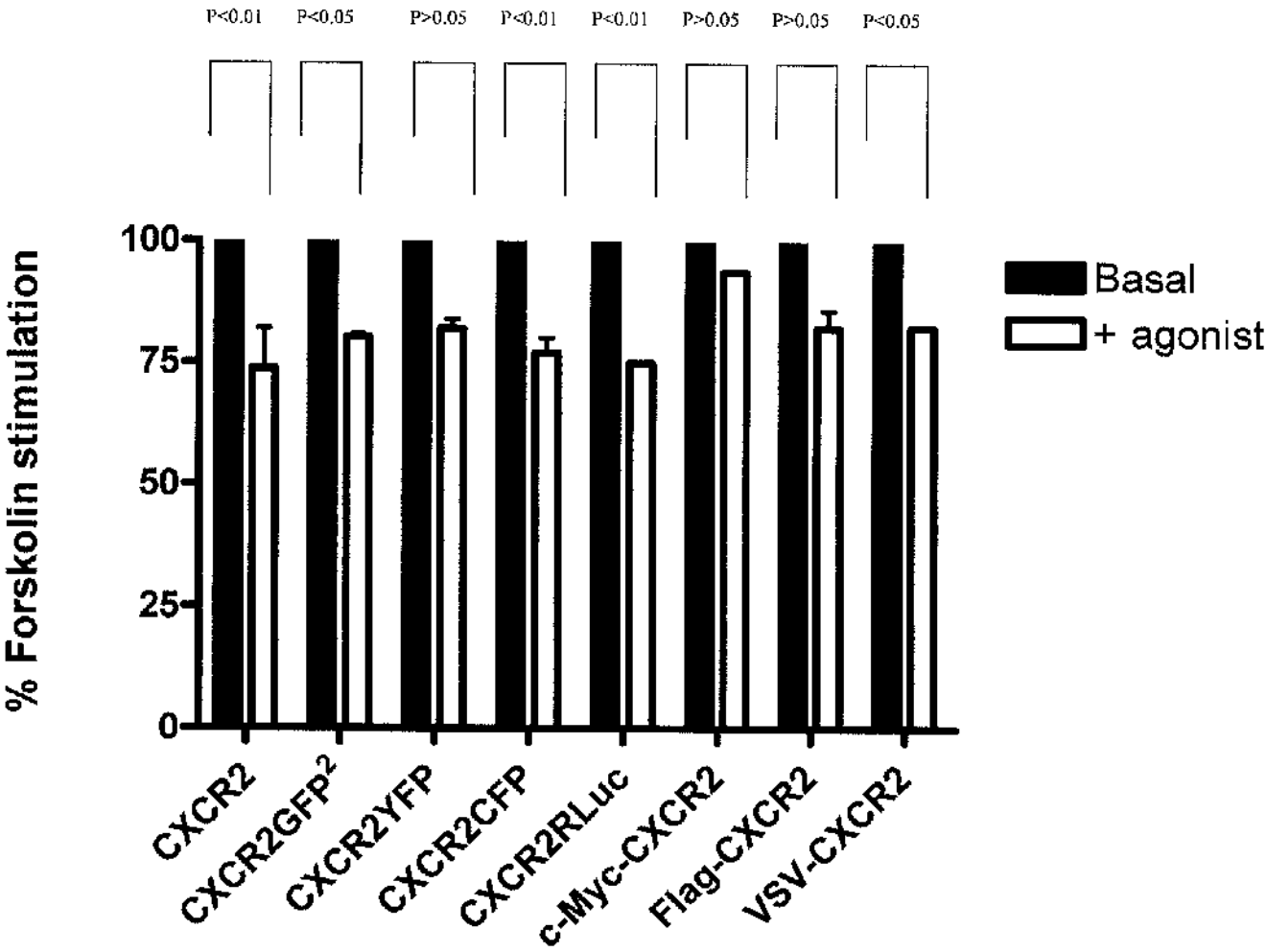


Figure 3.8 Agonist-induced inhibition of forskolin-induced cAMP generation by modified forms of CXCR2

Appropriately transfected cells were stimulated with 10 μ M forskolin and exposed to 100nM IL-8 for 30 minutes prior to cAMP detection using the DiscoverX XS cAMP kit. Data are expressed as percentage of forskolin induced cAMP release. Open bars represent cAMP released following exposure to forskolin (basal) while closed bars represent cAMP levels detected following exposure to IL-8 (+ agonist). Data shown are mean \pm S.E.M and representative of three individual experiments.

Figure 3.8



Chapter 4

4.1 Introduction

Oligomerisation within the chemokine receptor family was shown to have important physiological relevance with the discovery of a natural genetic mutation of CCR5, termed *ccr5-32Δ*, which conferred resistance to HIV-1 infection. Individuals homozygous for this mutation, approximately 1% of the population, were found to be resistant to HIV-1 infection (Liu *et al.*, 1996). Individuals heterozygous for this allele, up to 20% in some populations, displayed a delayed progression from HIV-1 to AIDS (Dean *et al.*, 1996; Samson *et al.*, 1996; Huang *et al.*, 1996; Michael *et al.*, 1997). The 32 base pair deletion in the open reading frame results in a frame shift premature stop codon within the third intracellular domain. This results in a truncated protein product that is not transported to the cell surface and thus cannot function as a co-receptor facilitating HIV-1 infection (Gharagozloo *et al.*, 2005). The delayed progression of infection observed in heterozygous individuals has been hypothesized to result from the homo-oligomerisation of *ccr5-32Δ* with wild type CCR5, resulting in a reduced level of CCR5 expressed at the cell surface (Benkirane *et al.*, 1997). However, Venkatesan *et al.*, (2002) demonstrated that the reduction in wild type CCR5 expression is a result of gene dosage rather than receptor sequestration. This study demonstrated that a molar excess of *ccr5-32Δ* does not significantly impair wild type CCR5 cell surface density when transiently transfected in HEK and Jurkat cells. The resistance to HIV-1 infection of *ccr5-32Δ* individuals has also been attributed to a gain-of-function effect. Agrawal *et al.*, (2004) found that *ccr5-32Δ* expression in primary CD4⁺ cells resulted in down-regulation of surface CXCR4 and wild-type CCR5, conferring resistance to HIV-1. CD4⁺ cells purified from individuals

homozygous for *ccr5-32Δ* also displayed lower levels of CXCR4. These findings have led to the hypothesis that *ccr5-32Δ* can alter expression of both CCR5 and CXCR4.

A polymorphism also exists for the CCR2 receptor in which valine at position 64 is replaced by isoleucine within the first transmembrane region, termed CCR2V64I. This mutation was shown to occur at an allelic frequency of 10-25% depending on the ethnic group of the population. Individuals possessing this polymorphism have demonstrated a delay in progression from HIV-1 to AIDS of 2-4 years (Smith *et al.*, 1997). Although CCR2 has not been shown to act as a co-receptor for HIV-1, it can form hetero-oligomers with CCR5 and CXCR4 (Mellado *et al.*, 1999). This is thought to form the basis of the delayed HIV-1 to AIDS progression. A recent study performed by Rodriguez-Frade *et al.*, (2004) utilised a monoclonal antibody directed against CCR2 to induce CCR2 hetero-oligomer formation with CCR5 or CXCR4. The authors showed that hetero-oligomer formation prevented HIV-1 entry via these receptors.

CXCR1 and CXCR2 have been implicated in the pathology of several disease states including chronic obstructive pulmonary disease and asthma (Owen, 2001). Interestingly, a non-competitive small molecule inhibitor of CXCR1 and CXCR2 has been developed to treat reperfusion injury occurring following organ transplant. The molecule acts by locking CXCR1 and CXCR2 in the inactive conformation preventing receptor signaling, chemotaxis and the release of inflammatory mediators. The drug named Repertaxin has proven to be well tolerated in animal tests and is progressing to phase I clinical trials (Casilli *et al.*, 2005). A recent study performed by Kaneider *et al.*, (2005) investigated the effect of pepducins directed against intracellular loops 1 and 3 of CXCR1 and CXCR2. The pepducins were found to prevent the IL-8 response of both receptors. IL-8 is a potent

pro-inflammatory mediator in sepsis and the intracellular loop peptides were shown to reverse sepsis when tested in mice. These studies clearly demonstrate the relevance of CXCR1 and CXCR2 in disease and the importance of further investigating interactions between these receptors.

These studies demonstrate the physiological relevance of chemokine receptor oligomerisation. The aim of the studies in this chapter was to investigate potential homo- and hetero-oligomerisation of CXCR1 and CXCR2. Several techniques were utilised including co-immunoprecipitation, Tr-FRET, FRET, and BRET. The selectivity of the observed oligomer formation was also investigated. The issue of ligand modulation of oligomerisation and the site of oligomer formation have been addressed. The internalisation properties of the observed hetero-oligomer were also studied.

4.2 Co-immunoprecipitation allows detection of CXCR1 and CXCR2 homo- and hetero-oligomers

In order to investigate the potential homo-oligomerisation of CXCR1, Flag- and c-Myc N-terminally tagged constructs were transiently expressed individually or co-expressed in HEK293T cells. Samples were solubilised by resuspending the transfected cells in 1x RIPA buffer. As a negative control, a mixed cell sample was included in which cells individually expressing the two constructs were mixed prior to immunoprecipitation. The cell lysates were incubated with anti-Flag antibody and SDS-PAGE performed on the precipitated material. Proteins were transferred onto nitrocellulose membrane and a western blot performed using anti-c-Myc antibody. Figure 4.1 demonstrates the co-immunoprecipitation of Flag and c-Myc-CXCR1. A band representing the 38kDa c-Myc-

CXCR1 receptor can be observed in the co-expressed sample, consistent with an interaction between Flag-CXCR1 and c-Myc-CXCR1. A band can also be observed at a slightly higher molecular mass, which may represent a differentially glycosylated form of the receptor. In addition a band at ~80kDa corresponding to the mobility of a homodimer was also detected, suggesting that a proportion of the immunoprecipitated oligomer was not completely separated by the SDS-PAGE conditions used. There was no immunoreactivity observed when each of the constructs was expressed alone. A control in which cell lysates corresponding to Flag-CXCR1 and c-Myc-CXCR1 were mixed prior to immunoprecipitation also did not demonstrate any immunoreactivity, confirming that expression of both epitope tagged receptors within the same cell is required to allow interaction. Cell lysates from the experiment were subjected to western blot analysis using anti-Flag and anti-c-Myc antibodies to ensure the correct expression of the constructs.

This protocol was repeated for Flag- and c-Myc-CXCR2 (Figure 4.2). Again this resulted in an immunoprecipitated band consistent with a complex of Flag-CXCR2/c-Myc-CXCR2. As observed for CXCR1, there was no immunoreactivity observed when each construct was expressed individually or cell lysates were mixed prior to immunoprecipitation. Consistent with results observed for CXCR1, immunoreactivity corresponding to a dimer was observed demonstrating that a fraction of the oligomeric complex was resistant to SDS-PAGE conditions.

The existence of a CXCR1/CXCR2 heterodimer was investigated. Flag-CXCR1 and c-Myc-CXCR2 were transiently transfected individually or co-expressed, and hetero-oligomerisation investigated as before. Figure 4.3 illustrates immunoreactivity representing c-Myc-CXCR2 immunoprecipitated using anti-Flag antibody when the two receptors have

been co-expressed, consistent with a Flag-CXCR1/c-Myc-CXCR2 complex. No immunoreactivity was observed in the other samples examined. The hetero-oligomer was also detected when Flag-CXCR2 and c-Myc-CXCR1 were co-expressed (data not shown).

4.3 Oligomerisation of CXCR1 and CXCR2 demonstrated using FRET in living cells

In order to investigate the existence of CXCR1 and CXCR2 oligomers in living cells, single cell FRET was used. Receptors were modified by attachment of either CFP or YFP at the C-terminus and expression of the modified receptors in HEK293T cells could be visualised (Figure 4.4). Expression of CXCR1CFP alone resulted in a FRET_{NORM} signal of 0.014 ± 0.002 . Upon co-expression of CXCR1CFP with CXCR1 fused to the acceptor protein YFP, a significant FRET_{NORM} signal of 0.93 ± 0.06 was detected. Previous studies performed within the Milligan group have demonstrated that co-expression of the isolated forms of CFP and YFP did not result in significant levels of FRET (Carrillo *et al.*, 2004). Donor and acceptor fluorescence levels were monitored to ensure relatively equal amounts, illustrated by the images shown.

The α_{1A} -adrenoceptor was used to investigate the specificity of the FRET signal detected. The expression pattern of this receptor does not overlap with either CXCR1 or CXCR2 and would not be predicted to interact with either receptor. Therefore the interactions between CXCR1/CXCR2 and α_{1A} -adrenoceptor were investigated as an indication of the selectivity of the observed oligomer formation. Co-expression of CXCR1CFP with α_{1A} -adrenoceptor-YFP yielded a FRET signal of 0.15 ± 0.03 demonstrating low energy transfer between the two receptors (Figure 4.4). Donor and acceptor expression levels were observed at similar levels to that observed for the CXCR1CFP/CXCR1YFP pairing, ensuring that the low

energy transfer observed was not an artefact of low acceptor expression.

CXCR2 homo-oligomerisation was also investigated using this method (Figure 4.5). Expression of CXCR2CFP alone resulted in a low energy transfer of 0.014 ± 0.001 . Co-expression of CXCR2CFP and CXCR2YFP resulted in strong energy transfer yielding a $\text{FRET}_{\text{NORM}}$ of 0.89 ± 0.08 . As with CXCR1, the interaction between CXCR2CFP and α_{1A} -adrenoceptor-YFP was investigated and resulted in a $\text{FRET}_{\text{NORM}}$ of 0.16 ± 0.07 demonstrating low levels of energy transfer between the two receptors. Images shown demonstrate the equal levels of donor and acceptor fluorescence when investigating oligomerisation.

Hetero-oligomerisation between CXCR1 and CXCR2 was investigated using this technique (Figure 4.6). CXCR1CFP and CXCR2YFP were co-expressed yielding a $\text{FRET}_{\text{NORM}}$ of 0.82 ± 0.04 . The opposite pairing, CXCR2CFP and CXCR1YFP resulted in energy transfer of 0.83 ± 0.05 . Results are shown in comparison to 'bleedthrough' resulting from expression of donor receptor alone into the FRET channel.

4.4 Oligomerisation of CXCR1 and CXCR2 investigated using BRET²

A modified version of bioluminescence resonance energy transfer termed BRET² was utilised to demonstrate the oligomerisation of CXCR1 and CXCR2 (Figure 4.7). The level of energy transfer was assessed by transfecting an equal amount of cDNA corresponding to receptor fused to *Renilla* luciferase and GFP² and measuring BRET² in intact cells. The level of apparent BRET² observed in cells transfected with the *Renilla* luciferase construct

alone was recorded to determine any 'bleed through' from the luciferase emission spectrum into the GFP² emission spectrum. Apparent BRET² resulting from expression of CXCR1Rluc alone yielded a ratio of 0.15 ± 0.003 while the co-expression of CXCR1 fused to both donor and acceptor moieties resulted in a BRET² ratio of 0.4 ± 0.01 demonstrating energy transfer. Consistent with FRET experiments, energy transfer between CXCR1 and the α_{1A} -adrenoceptor was studied to investigate the specificity of oligomer formation. Co-expression of α_{1A} -adrenoceptor-GFP² with CXCR1Rluc yielded a BRET² ratio of 0.18 ± 0.003 . This was repeated using CXCR2Rluc and GFP². Expression of CXCR2Rluc alone resulted in an apparent BRET² ratio of 0.2 ± 0.001 while the co-expression of CXCR2Rluc and CXCR2GFP² yielded robust energy transfer of 0.5 ± 0.005 . Co-expression of CXCR2Rluc with α_{1A} -GFP² resulted in a BRET² ratio of 0.2 ± 0.002 .

Hetero-oligomerisation of CXCR1 and CXCR2 was assessed using this technique. Co-expression of CXCR1Rluc and CXCR2GFP² resulted in a BRET² ratio of 0.5 ± 0.004 while the reciprocal pairing yielded a ratio of 0.4 ± 0.014 .

In order to investigate the propensity of the homo- and hetero- oligomers to form, BRET² was used in saturation BRET² experiments. Saturation BRET² experiments have been extensively used to investigate the propensity of a given receptor pair to form oligomers (Mercier *et al.*, 2002). BRET² is measured in cells expressing varying ratios of acceptor protein to donor protein allowing BRET² saturation curves to be constructed. BRET_{MAX} is a function of the total number of oligomers formed and the distance and orientation of the donor and acceptor moieties to each other. BRET₅₀ values are an indication of the relative

affinity of the receptor conjugated to the receptor for the receptor conjugated to the donor moiety (Mercier *et al.*, 2002). BRET² ratios were corrected for apparent BRET² resulting from 'bleedthrough' of CXCR1Rluc and CXCR2Rluc into the GFP² channel. This permits the determination of the relative affinity of the interaction at half-maximal signal. Following expression of a constant amount of CXCR1-RLuc with an increasing amount of CXCR1-GFP², addition of DeepBlueC resulted in BRET² signals which generated a hyperbolic curve approaching an asymptote with increasing [acceptor/donor] ratios yielding a BRET_{MAX} of 0.28±0.05 and a BRET₅₀ of 3.6±0.4 (Figure 4.8). Potential energy transfer between CXCR1Rluc and GFP² vector was used as a control in this experiment and was found to yield very low levels of energy transfer demonstrating that the energy transfer observed did not result from a mutual affinity between donor and acceptor moieties. Analysis of the data demonstrated that it generated a linear relationship with increasing [A]/[D] ratios, inferring that the interaction was non-specific.

The characteristics of the CXCR2 oligomer were then investigated. Again a constant amount of CXCR2Rluc was co-expressed with increasing amounts of CXCR2GFP² and the BRET² signal recorded following the addition of the substrate. As can be observed in Figure 4.8 the CXCR2 homo-oligomer pairing yielded a BRET_{MAX} of 0.36±0.03 and BRET₅₀ of 2.2±0.1 demonstrating that high affinity interactions occur, similar to that observed for CXCR1. In order to investigate the specificity of the homo-oligomer interaction, the interaction of CXCR2Rluc and α_{1A} -adrenoceptor-GFP² was investigated. BRET² signals generated by this pairing were analysed using statistical comparison of model fitting. This yielded r^2 values of 0.95 and 0.90 for linear and non-linear models respectively. This indicates that a linear model is the best fit with BRET² ratios

proportional to [A]/[D] levels. This suggests that the pair had low affinity for each other. Hetero-oligomerisation between CXCR1 and CXCR2 was then investigated (Figure 4.8). This yielded a $BRET_{MAX}$ of 0.42 ± 0.04 and $BRET_{50}$ of 3.6 ± 0.1 .

The ability of ligand to modulate oligomerisation was investigated using $BRET^2$. Intact cells transfected with an equal amount of donor and acceptor cDNA were incubated with 50nM IL-8 for 30 minutes at 37°C before the addition of DeepBlueC substrate. The results presented in Figure 4.9 demonstrate that the exposure of ligand did not cause any significant alteration in the $BRET^2$ levels detected.

4.5 Oligomerisation of CXCR1 and CXCR2 demonstrated using Tr-FRET

Tr-FRET was utilised to investigate if chemokine receptor oligomers exist pre-formed at the cell surface. Both FRET and BRET can report energy transfer occurring throughout the cell therefore it is important to determine if the observed receptor oligomers are present at the cell surface. Firstly Flag- and c-Myc-CXCR1 and CXCR2 were expressed transiently in HEK293T cells. As a control mixed cells expressing the differentially tagged constructs individually were also used. In order to investigate the specificity of this energy transfer the α_{1A} adrenoceptor was selected as a control. Cells were incubated with anti-c-Myc antibody conjugated to Europium (Eu^{3+}) that acts as the energy donor and anti-Flag antibody conjugated to allophycocyanin (APC) as the energy acceptor. Figure 4.10 demonstrates the strong energy transfer signal observed for CXCR1 homo-oligomers yielding a Tr-FRET ratio of 0.062 ± 0.004 . Very low energy transfer of -0.001 ± 0.001 for CXCR1 was observed for mixed cells individually expressing the constructs. When the c-

Myc- α_{1A} -adrenoceptor construct was co-transfected with Flag-CXCR1, a Tr-FRET ratio of 0.026 ± 0.003 was obtained. This was repeated using Flag- α_{1A} -adrenoceptor and c-Myc-CXCR1 yielding a Tr-FRET ratio of 0.03 ± 0.01 . As previously, the mixed cell controls for these combinations demonstrated a negligible amount of Tr-FRET.

A slightly stronger energy transfer was observed for the CXCR2 homo-oligomer of 0.075 ± 0.005 as demonstrated in Figure 4.11. The mixed cell control presented a very low energy transfer ratio of 0.001 ± 0.001 . Again using the c-Myc- α_{1A} -adrenoceptor construct to investigate specificity of the interaction, the Flag-CXCR2 and c-Myc- α_{1A} -adrenoceptor combination yielded a value of 0.026 ± 0.002 . This was repeated using Flag- α_{1A} -adrenoceptor and c-Myc-CXCR2 obtaining 0.04 ± 0.01 . The mixed cell controls for these pairs again indicated very low levels of energy transfer.

Hetero-oligomerisation was investigated using this technique (Figure 4.12). A robust energy transfer was observed for the Flag-CXCR1/c-Myc-CXCR2 hetero-oligomer presenting a Tr-FRET ratio of 0.07 ± 0.02 . As had been observed previously, the mixed cell control yielded very low energy transfer of 0.002 ± 0.002 for this pairing. The alternative combination of tagged receptors was tested using c-Myc-CXCR1 and Flag-CXCR2. The pairing demonstrated similar high levels of Tr-FRET presenting a ratio of 0.06 ± 0.01 . The mixed cell controls for these combinations yielded negligible Tr-FRET ratios.

As this technique only detects cell surface oligomerisation, it is a useful tool when investigating ligand modulation of oligomers. Cells were incubated with 100nM IL-8 for 15 minutes. Figure 4.13 clearly demonstrates that agonist had no effect on the levels of Tr-FRET observed for the homo-oligomers. This protocol was repeated to investigate ligand

modulation of the hetero-oligomer with no detectable change in the energy transfer observed (Figure 4.13).

4.5 Oligomerisation of CXCR1 and CXCR2 investigated using ER retention assay

In order to determine the location of the oligomer formation, an ER retention assay was developed. This assay system utilised an approach that had been previously used to investigate the formation of K^{ATP+} channel complexes (Schwappach *et al.*, 2000). These channels are multimeric complexes containing an ER retention sequence that is masked upon complex formation (Schwappach *et al.*, 2000). This study described a trafficking trap assay, reasoning that if one subunit in a receptor complex contains an ER retention sequence that is not hidden by co-assembly, then any interacting protein will also be trapped within the ER. Therefore, if a protein that is normally membrane expressed is retained in the ER, it must be interacting with the protein containing the strong retention signal. The retention signal used in this study was derived from the C-terminal of the α_{2C} -adrenoceptor that has been shown to contain an ER retention motif (Zerangue *et al.*, 1999; Margeta-Mitrovic, 2002). HA-CXCR1 was modified by the addition of 14 amino acids encompassing the ER retention sequence onto the C-terminal tail of the receptor, to produce the construct termed HA-CXCR1-ER.

In order to investigate the distribution pattern of the wild type receptor, unmodified CXCR1 was transiently expressed in HEK293T cells and subjected to immunocytochemistry using an anti-CXCR1 antibody on both intact and permeabilised cells. As shown in Figure 4.14 (a), staining of intact and permeabilised cells demonstrated that the majority of the receptor was clearly membrane delineated with very little

intracellular staining. An N-terminally HA-tagged form of the α_{2C} -adrenoceptor was transiently expressed in HEK293T cells (Figure 4.14 (b)) and immunocytochemistry on intact cells using an antibody directed against the HA epitope tag, revealed a small degree of surface staining while analysis of permeabilised cells revealed a strong signal corresponding to receptors retained in vesicles in the cell. This is consistent with data showing that the α_{2C} -adrenoceptor, when expressed in HEK293 cells, was localised in intracellular compartments with little membrane expression observed (Daunt *et al.*, 1997). Examination of HA-CXCR1-ER that contains the retention sequence C-terminally fused also demonstrated a similar pattern of intracellular retention as the HA- α_{2C} -adrenoceptor upon staining of permeabilised cells. Very little membrane expression of HA-CXCR1-ER was observed following examination of intact cells (Figure 4.14 (c)).

The overlapping expression of c-Myc CXCR1 and HA-CXCR1-ER was investigated. As demonstrated in chapter three, c-Myc-CXCR1 is cell surface expressed however upon co-expression with HA-CXCR1-ER, permeabilisation of cells prior to immunocytochemistry reveals an overlapping expression pattern of intracellular receptor staining (Figure 4.15 (a)). This suggests an interaction is occurring between c-Myc-CXCR1 and HA-CXCR1-ER resulting in the intracellular retention of c-Myc-CXCR1. Similar results were obtained for the co-expression of c-Myc-CXCR2 and HA-CXCR1-ER (b).

The ability of the receptor containing the retention sequence to prevent cell surface expression of other receptors was assessed (Figure 4.16). Cell surface expression of Flag-CXCR1 was quantified using whole cell immunostaining following co-transfection with either HA-CXCR1 or HA-CXCR1-ER. There was a significant reduction in cell surface

expression of Flag-CXCR1 observed upon co-expression with HA-CXCR1-ER. A similar reduction in cell surface expression was shown for Flag-CXCR2 upon co-expression with HA-CXCR1-ER. In order to investigate the selectivity of the interaction, the cell surface expression of Flag- α_{1A} -adrenoceptor was examined upon co-transfection with HA-CXCR1 or HA-CXCR1-ER. There was no significant difference in the amount of Flag- α_{1A} -adrenoceptor expression at the cell surface when expressed with HA-CXCR1-ER in comparison to expression with HA-CXCR1, consistent with a lack of specific interaction between CXCR1 and α_{1A} -adrenoceptor.

4.6 Do CXCR1 and CXCR2 co-internalise?

As detailed in section 1.2.5.1, there have been several reports detailing that GPCR hetero-oligomers can undergo internalisation in response to an agonist directed against only one component of the hetero-oligomer complex. Tr-FRET data demonstrated the existence of CXCR1/CXCR2 hetero-oligomers at the cell surface. The ability of the hetero-oligomer complex to undergo co-internalisation was investigated. c-Myc-CXCR1 and VSV-CXCR2 were transiently co-expressed in HEK293T cells grown on coverslips. Immunocytochemistry was performed to visualise the receptors in the absence and presence of 50nM IL-8, a ligand with high affinity for both CXCR1 and CXCR2, and 100nM GRO- α , which has high affinity for CXCR2 but low affinity for CXCR1. Figure 4.17 demonstrates that in non-stimulated cells both c-Myc-CXCR1 (red) and VSV-CXCR2 (green) were clearly membrane delineated. Upon stimulation with IL-8, both c-Myc-CXCR1 and VSV-CXCR2 undergo internalisation into punctate vesicles that were found to co-localise yielding a yellow colour in the overlay image. Upon stimulation with GRO- α , VSV-CXCR2 underwent internalisation into punctate vesicles however Myc-CXCR1

remained membrane localised. This suggests that the CXCR1/CXCR2 hetero-oligomer does not undergo co-internalisation with only the CXCR2 homo-oligomers internalising in response to GRO- α .

4.7 Discussion

As discussed previously several members of the class A GPCR family have been shown to form homo- and hetero-oligomers. In this study the formation of constitutive CXCR1 and CXCR2 homo-oligomers and CXCR1/CXCR2 hetero-oligomers has been demonstrated using several different techniques. These receptor homo- and hetero-oligomers have been shown to exist pre-formed at the cell membrane. Investigation into the site of oligomer formation demonstrated that the receptors form oligomers during protein synthesis and maturation. The selectivity of the oligomer formation was shown by the lack of substantial interactions between the α_{1A} -adrenoceptor and CXCR1 or CXCR2.

A co-immunoprecipitation approach was used in this study. This is a technique that requires cell lysis and solubilisation, a procedure that could result in artefactual results being observed due to the highly hydrophobic nature of GPCRs (Salim *et al.*, 2002). However, the inclusion of a mixed cell control in which cells expressing each epitope tagged receptor alone were mixed prior to immunoprecipitation served to address this caveat. This control was performed in my studies but yielded no immunoreactivity, demonstrating that the positive result observed was not an artefact of the experimental protocol. Figures 4.1, 4.2 and 4.3 show that a proportion of the immunoprecipitated oligomer was not fully separated by the SDS-PAGE conditions used as immunoreactivity corresponding to the predicted molecular mass of both the monomer and dimer of CXCR1

and CXCR2 was observed. This observation suggests that a fraction of the CXCR1 and CXCR2 homo- and hetero-oligomers demonstrated in this study exist as oligomers using non-covalent interactions. However, it is possible that the levels of homo- and hetero-oligomers formed in these experiments were very high and therefore the reducing conditions employed may have been insufficient to fully reduce all oligomers. A recent study investigating oxytocin receptor oligomerisation demonstrated that at low levels of receptor expression the formation of SDS-resistant oligomers is favoured while at higher levels of receptor expression of 400 fmol/mg and above, the formation of SDS-sensitive oligomers are observed (Devost and Zingg, 2004).

The presence of both SDS-sensitive and resistant CXCR2 homo-oligomers has been observed previously (Trettel *et al.*, 2003). This study utilised a co-immunoprecipitation approach to demonstrate the existence of constitutive CXCR2 homo-oligomers in HEK293 cells and rat cerebellar granule neurons. Interestingly the authors did not observe CXCR1 homo-oligomer formation or indeed CXCR1/CXCR2 hetero-oligomer formation, both of which have been shown in this study using several techniques.

Single cell FRET is a RET technique that allows protein: protein interactions to be recorded in living cells. This technique has the advantage of allowing the relative ratios of donor and acceptor fluorescent moieties to be defined in order to ensure they are comparable. This permits the comparison of FRET signals generated from different GPCR combinations by excluding the factor of receptor expression levels influencing energy transfer. As can be observed in Figures 4.4, 4.5 and 4.6, constitutive homo- and hetero-oligomerisation was observed for CXCR1 and CXCR2. This positive FRET signal is not a result of mutual affinity between the donor and acceptor fluorescent proteins as a study

performed by Carillo *et al.*, (2004) demonstrated that no significant amount of FRET was detected upon co-expression of isolated forms of CFP and YFP. A low FRET signal was observed between CXCR1/2 and α_{1A} -adrenoceptor. The cell images shown demonstrate that the relative levels of donor and acceptor fluorescent conjugates in these experiments were similar. This suggests that the low FRET levels reported are a reflection of a lack of interaction as opposed to an artefact resulting from insufficient acceptor levels to produce a FRET signal. In order to confirm energy transfer was occurring between the two receptor-fluorophore conjugates, photo-bleaching FRET could be employed. As mentioned previously, this technique involves images of donor fluorescence compared before and after the prolonged excitation of the acceptor moiety resulting in irreversible photochemical destruction. An increase in donor fluorescence following destruction of the acceptor protein indicates energy transfer occurring between the donor and acceptor protein conjugates.

Saturation BRET is an application of BRET that permits the determination of relative affinities of the donor and acceptor receptor constructs for each other. This is achieved by exploiting the theory that upon expression of a constant amount of donor and an increasing amount of acceptor, energy transfer should increase until all donor molecules are interacting with acceptor molecules. This results in a plateau after which increasing concentrations of acceptor has no further effect on the energy transfer levels observed (Mercier *et al.*, 2002). Theoretically, specific interactions between donor and increasing concentrations of acceptor should result in BRET signals permitting generation of a saturation curve. BRET signals resulting from the random collision of donor and acceptor moieties are predicted to result in the formation of a quasi-linear relationship between BRET signal and acceptor concentration. Several studies have utilised this approach to

generate BRET₅₀ values, indicating the selectivity of the oligomer formation (Canals *et al.*, 2004; Canals *et al.*, 2003; Mercier *et al.*, 2002; Breit *et al.*, 2004; Ayoub *et al.*, 2004; Terrillon *et al.*, 2003). In this study the relative affinities of the CXCR1 and CXCR2 homo- and hetero-oligomers are very similar suggesting that they have an equal propensity to form homo- and hetero-oligomers. As mentioned previously, neutrophils were found to express equal amounts of CXCR1 and CXCR2, however, monocytes and positive lymphocytes express more CXCR2 than CXCR1 (Chuntharapai *et al.*, 1994). This could have a potential implication on the theoretical equilibrium that exists between homo- and hetero-oligomer formation under physiological conditions. The excess of CXCR2 receptor expression in comparison to CXCR1 receptor levels found in monocytes and positive lymphocytes could lead to preferential formation of hetero-oligomers in these cells.

Values obtained corresponding to BRET_{MAX} for CXCR2 homo-oligomers and CXCR1/CXCR2 hetero-oligomers were higher than those values observed for CXCR1 homo-oligomer formation. This is likely to be the result of the orientation of donor and acceptor moieties within CXCR1 homo-oligomers being less conducive to energy transfer than observed for CXCR2 homo-oligomers and CXCR1/CXCR2 hetero-oligomer formation. Also the interaction observed between CXCR2Rluc and α_{1A} -adrenoceptor-GFP² yielded BRET signals that increased linearly with increasing amounts of acceptor, suggesting that the energy transfer was the result of random interactions. This was also observed for CXCR1Rluc and GFP² vector alone albeit with lower BRET values obtained.

RET techniques have been criticised in the past due to the high levels of receptor expression commonly used in these experiments. Protein over-expression could potentially result in energy transfer occurring as a result of random interactions and not from oligomer

formation. A study performed by Mercier *et al.*, (2002) investigated the impact of receptor expression levels on energy transfer observed for the β_2 -adrenoceptor. BRET was recorded from a range of samples expressing receptors at levels ranging from 1.4 to 87.2 pmol/mg. For protein expression between 1.4 - 26.3 pmol/mg, the energy transfer that occurred between donor and acceptor moieties did not increase. However, at receptor densities of 47.3 pmol/mg and above, the BRET signal increased. This could be due to artefactual aggregation caused by the high expression levels used or random interactions between donor and acceptor molecules already engaged in dimers. Similar experiments have been performed for CCR5, CXCR4 and opioid receptors yielding data indicating that at relatively low expression levels, BRET is independent of receptor density (Issafras *et al.*, 2002; Babcock *et al.*, 2003). In this study protein expression levels were not monitored due to experimental difficulties in obtaining reliable receptor expression data. Further evidence supporting the independence of RET from receptor expression levels was reported by Terrillon *et al.*, (2003). This study fractionated transiently transfected cells expressing BRET donor and acceptor protein conjugates on the basis of YFP expression. The subpopulations of cells were then examined and BRET recorded. It was found that energy transfer observed for these fractions did not differ significantly from that observed for the whole cell population, supporting the theory that maximal RET levels are independent of receptor density.

The results gained from Tr-FRET demonstrated that CXCR1 and CXCR2 homo- and hetero-oligomers exist at the cell surface. However, the data yielded from these experiments when investigating the interaction of CXCR1 or CXCR2 with α_{1A} -adrenoceptor was suggestive of a degree of oligomerisation occurring between the two receptors. This is in disagreement with previous data generated from FRET and BRET

studies in which the co-expression of CXCR1/CXCR2 and α_{1A} -adrenoceptor donor and acceptor conjugates yielded low energy transfer and, most convincingly, BRET saturation analysis demonstrated the non-specific nature of these interactions. Both orientations of CXCR1/CXCR2 and α_{1A} -adrenoceptor were tested in that Tr-FRET was recorded when the α_{1A} -adrenoceptor was N-terminally tagged with either c-Myc or Flag epitope tags, corresponding to the donor and acceptor antibodies respectively. Figure 4.10 and 4.11 demonstrate that Tr-FRET levels were increased when the Flag epitope tagged form of the α_{1A} -adrenoceptor was used. As mentioned previously energy transfer is an acceptor dependent process and it is possible that in these experiments the levels of expression of the N-terminally Flag tagged α_{1A} -adrenoceptor acting as an energy acceptor exceeded CXCR1 and CXCR2 receptor expression levels, resulting in increased energy transfer observed. This is suggested by the control experiments detailed in Figure 4.10 (b) and 4.11 (b) demonstrating higher levels of Flag-APC fluorescence corresponding to Flag- α_{1A} -adrenoceptor than observed for Flag-CXCR1/2. Receptor expression levels were not monitored in this study and it is possible that expression of the α_{1A} -adrenoceptor reached high levels that would permit Tr-FRET irrespective of whether an interaction was actually occurring.

Tr-FRET utilises antibodies raised to N-terminally expressed epitope tags that are conjugated to suitable donor and acceptor moieties. The possibility of antibody binding causing receptor aggregation and therefore generating a false positive result has been suggested (McVey *et al.*, 2001). However, although the antibodies used are bivalent, antibody-induced receptor clustering would only induce dimer formation between receptors expressing the same epitope tag. Tr-FRET reports on energy transfer between two differentially epitope tagged receptors, therefore any clustering effect would be

predicted to have no influence on the energy transfer reported in this assay. This is confirmed in Figures 4.10, 4.11 and 4.12 in which a control composed of cells individually expressing differential epitope tags mixed prior to antibody incubation was included, yielding very low levels of energy transfer. If the antibodies were to induce receptor aggregation leading to false positive results, then Tr-FRET should have been observed in these controls.

Recently several studies have addressed the possibility that energy transfer could be a result of receptor aggregation in membrane microdomains yielding an artefactual result rather than resulting from oligomer formation. Zacharias *et al.*, (2002) highlighted that energy transfer between closely located receptor molecules can occur even when they are not directly interacting when expressed in heterologous expression systems. Localisation of receptors within membrane rafts would permit close association of receptors (<10nm), facilitating energy transfer. Canals *et al.*, (2003) utilised methyl- β -cyclodextrin treatment in order to demonstrate that the disruption of membrane rafts by this chemical had no effect on the BRET signal observed corresponding to adenosine A_{2A} and dopamine D2 hetero-oligomers. A similar protocol was used to investigate membrane raft contribution to adenosine A_{2A} homo-oligomer formation (Canals *et al.*, 2004). Following methyl- β -cyclodextrin treatment, cells were repleted with cholesterol but this also had no effect on the BRET signal observed. A dominant negative mutant of dynamin was used to investigate the formation of TRH receptor aggregates within clathrin-coated pits (Kroeger *et al.*, 2001). Expression of this mutant had no effect on the levels of BRET recorded. Although the possibility of receptor aggregation in membrane microdomains was not addressed directly in this study, any RET resulting from artefactual results should have been observed between CXCR1/CXCR2 and α_{1A} -adrenoceptor assuming they are directed

to the same micro-domains. As can be observed in Figures 4.4, 4.5 and 4.8, the levels of energy transfer observed were substantially less than observed for either CXCR1/CXCR2 homo- or hetero-oligomerisation. Also, a recent study performed by Rose *et al.*, (2004) demonstrated that CXCR1 and CXCR2 were excluded from Triton-X-100 insoluble lipid rafts, both at basal conditions and following chemokine treatment, thus reducing further the likelihood of energy transfer resulting from receptor aggregation within these domains.

In this study incubation with IL-8 had no significant effect on energy transfer observed in either BRET or Tr-FRET. This is consistent with a study performed by Trettel *et al.*, (2003) in which incubation with ligand had no effect on CXCR2 oligomerisation detected using co-immunoprecipitation. Ligand-dependent increases in energy transfer have been reported for CXCR4 and CCR5 (Toth *et al.*, 2004; Hernanz-Falcon *et al.*, 2004). For CXCR4, binding of the CXCR4 antagonist AMD3100 was able to attenuate the ligand-induced increase in energy transfer, leading to the hypothesis that chemokine receptors exist in an equilibrium of monomers and higher order oligomers that is influenced by ligand binding (Toth *et al.*, 2004). The effects of IL-8 on BRET were assessed using cells transfected with a ratio of donor and acceptor yielding energy transfer at a level close to BRET_{MAX}. It could be possible that the orientation of donor and acceptor moieties at BRET_{MAX} do not permit any small changes in energy transfer resulting from ligand binding to be observed. However, Canals *et al.*, (2004) investigated BRET signals following agonist exposure to adenosine A_{2A} receptor homo-oligomers at donor: acceptor ratios corresponding to minimum, half-maximum and maximum BRET signals and observed no alteration in energy transfer. Detecting ligand-induced changes in BRET is complicated by the fact that the *Renilla* luciferase substrate DeepBlueC is cell permeable and therefore the energy transfer represents donor and acceptor interactions throughout the

cell. As the ligand was incubated with whole cells, only receptor oligomers that are surface expressed will bind ligand and any modulation in BRET signal may not be discernable as the signal is representative of energy transfer occurring throughout the cell. As demonstrated in chapter 3, the majority of the modified CXCR1 and CXCR2 are cell surface expressed with only a small proportion observed within the cell. However, it is possible that energy transfer could be observed between receptors that have been incorrectly folded and retained within the cell and could influence any significant modulation of energy transfer observed following ligand binding. In order to address this problem associated with BRET, Tr-FRET was used. This technique, when performed in whole cells, only reports Tr-FRET occurring at the cell surface therefore eliminating any contribution to energy transfer resulting from interactions within the cell. In this study incubation with IL-8 had no effect on the Tr-FRET signal observed for CXCR1 and CXCR2 homo- and hetero-oligomers. For CXCR1/CXCR2 hetero-oligomer formation, both orientations of donor and acceptor were tested to exclude the possibility that the different receptor-donor/acceptor conjugate pairings conferred a conformational arrangement permitting detection of any ligand-induced modulation. The lack of ligand modulation of CXCR1 and CXCR2 oligomerisation is consistent with several reports investigating oligomerisation of several class A GPCRs detailed in section 1.2.4.1.

An ER retention assay was developed in this study to demonstrate that homo-oligomerisation of CXCR1 and hetero-oligomerisation of CXCR1/CXCR2 occurs in the ER. As described in section 1.2.4.2 several studies have demonstrated the presence of GPCR oligomers in the ER (Overton *et al.*, 2002; Issafras *et al.*, 2002; Terrillon *et al.*, 2003). The assay developed in this study utilised a form of CXCR1 that was C-terminally modified to express an ER retention sequence derived from the α_{2C} -adrenoceptor. This

modified form of CXCR1 was capable of acting as a dominant negative on wild type CXCR1 and CXCR2, inhibiting normal cell surface expression. This suggests that CXCR1 homo-oligomerisation and CXCR1/CXCR2 hetero-oligomerisation occurs in the ER. This is consistent with observations made by Trettel *et al.*, (2003) who demonstrated that for CXCR2, homo-oligomers were still detected in the absence of glycosylated monomeric receptors. This suggests that oligomerisation occurred prior to plasma membrane delivery and at some point during the biosynthetic pathway. Retention of wild type receptor by intracellularly retained naturally occurring mutant forms has been demonstrated physiologically for the CCR5 receptor. A mutation exists that is not cell surface expressed and serves to reduce wild type cell surface expression by hetero-oligomer formation (Benkirane *et al.*, 1997).

There is a possibility that export from the ER could be inhibited because accumulation of the retained form of CXCR1 could potentially cause congestion of the secretory pathway. This could result in the retention of wild type receptor regardless of whether the ER retained form of CXCR1 and the wild type receptor are interacting. However, if this was the case a reduction in cell surface expression should also have been observed for the co-expression of CXCR1 possessing the ER retention motif and the α_{1A} adrenoceptor, which was not observed.

Recently oligomer formation has been suggested to be necessary for targeting of GPCRs from the ER to the plasma membrane. This has been shown for the β_2 -adrenoceptor where mutations of a previously identified putative oligomerisation motif present in TMVI (Hebert *et al.*, 1996) perturbed receptor cell surface targeting (Salahpour *et al.*, 2004). This has also been shown for the yeast α factor receptor by targeting an oligomerisation motif

present in TMI (Overton *et al.*, 2003). However, recent investigation into CCR5 oligomerisation demonstrated that two amino acid substitutions performed in TMI and IV were sufficient to abrogate receptor function and oligomerisation detected by FRET but were cell surface expressed (Hernanz-Falcon *et al.*, 2004). This result suggests that oligomerisation does not have a role in CCR5 export from the ER. It could be suggested that these mutations have simply resulted in a conformation of oligomers in which the orientation of donor and acceptor moieties is not permissive to energy transfer, however further studies would be required in order to confirm this theory.

As CXCR1 and CXCR2 hetero-oligomers were shown to exist constitutively at the cell membrane, hetero-oligomer function was investigated. As discussed in section 1.2.5.1, the ability of hetero-oligomers to undergo internalisation in response to an agonist directed at only one receptor within the oligomer complex has been shown for several receptor pairs (Rocheville *et al.*, 2000; Xu *et al.*, 2003; Stanasila *et al.*, 2003; Jordan *et al.*, 2001; Pfeiffer *et al.*, 2002; Hillion *et al.*, 2002). In this study N-terminally tagged c-Myc-CXCR1 and VSV-CXCR2 were co-expressed and stimulated with the agonist GRO- α , a CXCR2 agonist with only weak activity at CXCR1. Co-internalisation was not observed as VSV-CXCR2 clearly underwent internalisation into punctate intracellular vesicles while c-Myc-CXCR1 remained membrane delineated. This observation could be due to preferential formation of CXCR2 homo-oligomers rather than CXCR1/CXCR2 hetero-oligomers, and subsequent visualisation of CXCR2 homo-oligomer internalisation. However, the BRET² saturation experiments performed in section 4.8 demonstrated highly similar propensities of homo-oligomer and hetero-oligomer formation, suggesting this is not the case. It is also conceivable that the CXCR1/CXCR2 hetero-oligomer may be resistant to agonist-induced internalisation. As both CXCR1 and CXCR2 undergo prominent agonist-induced

internalisation in response to IL-8 when coexpressed, the lack of hetero-oligomer co-internalisation cannot be attributed to one component of the complex that is poorly internalised exerting a dominant negative effect on the other component. Upon ligand activation the hetero-oligomer may dissociate into its monomeric state. This possibility is not supported by the BRET and Tr-FRET data that reported no change in energy transfer levels following exposure to agonist, interpreted as no change in oligomer levels. Hetero-oligomerisation of the DOP and β_2 -adrenoceptor has been demonstrated (McVey *et al.*, 2001; Jordan *et al.*, 2001). However, a study performed by Tsao *et al.*, (2000) reported that when β_2 -adrenoceptor and DOP were stably co-expressed, internalisation of each receptor was achieved only upon exposure to the receptor specific agonist. The study continued to resolve the fate of the receptors following endocytosis. Despite utilising the same endocytic pathway, the receptors were differentially targeted to recycling and degradation pathways. Recently a study performed by El-Asmar *et al.*, (2005) described CCR2/CCR5 hetero-oligomer formation. The authors utilised BRET saturation experiments to demonstrate that hetero-oligomer and homo-oligomer formation occurred with similar propensity, however, co-internalisation of the CCR2/CCR5 complex was not observed using internalisation assays. This is obviously an area that requires further investigation to accurately determine the mechanisms regulating co-internalisation.

As mentioned previously, studies performed investigating chemokine receptor oligomerisation have generated conflicting results. Initial reports suggested that oligomerisation of chemokine receptors was a ligand-dependent process (Rodriguez-Frade *et al.*, 1999; Vila-Coro *et al.*, 2000; Vila-Coro *et al.*, 1999; Mellado *et al.*, 2001). However, several reports have now shown constitutive chemokine receptor oligomerisation consistent with the data in this study (Babcock *et al.*, 2003; Issafras *et al.*, 2002 Trettel *et*

al., 2003; Toth *et al.*, 2004; Hernanz-Falcon *et al.*, 2004). A pattern governing the oligomerisation of these receptors is difficult to observe at the present time.

CXCR2 oligomerisation has previously been demonstrated (Trettel *et al.*, 2003). This study demonstrated the existence of CXCR2 oligomers expressed in HEK cells and cerebellar neuron cells. Co-immunoprecipitation of differentially tagged forms of the CXCR2 receptor was also shown. However, this group did not report CXCR1 homo-oligomerisation or CXCR1 and CXCR2 hetero-oligomerisation. The experimental protocol detailed in the study did not differ significantly from the methods employed in this study and very little detail was given relating to the experiments investigating CXCR1 homo- and hetero-oligomerisation. Therefore, it is difficult to identify a reason for the conflicting results.

The mechanisms of chemokine receptor oligomerisation have been studied. Trettel *et al.*, (2003) utilised N- and C- terminal truncations of the CXCR2 receptor to identify a region potentially involved in oligomerisation. Results demonstrated that a region between Ala-106 and Lys-163, predicted to contain the first extracellular loop, TM III and the second intracellular loop, was required for CXCR2 homo-oligomer formation detected by co-immunoprecipitation. The authors also interpreted the presence of SDS-resistant CXCR2 oligomers as support that TMIII is involved in oligomer formation. As mentioned previously two amino acid substitutions, I52V and V150A, located in TMI and IV of CCR5 resulted in a non-functional receptor that was unable to form oligomers when assessed by FRET (Hernanz-Falcon *et al.*, 2004). The location of these point mutations was the result of bioinformatic analysis that predicted that these regions are important in oligomerisation. Amino acid substitution in CCR2 of residues V64A and V164A in TM I

and IV respectively also resulted in a non-functional form of the receptor than could not form oligomers when assessed by FRET. The amino acids predicted to be involved in oligomerisation are receptor specific. The regions containing the amino acids mutated in this study are present in motifs conserved across the chemokine receptor family, indicating that these regions could be important in chemokine receptor oligomerisation. However, recently Lcmay *et al.*, (2005) have attempted to utilise the mutated forms of CCR5 and investigate oligomerisation using co-immunoprecipitation and BRET. This group demonstrated that oligomerisation could still be detected in those receptors possessing these mutations. Hernanz-Falcon *et al.*, responded by stating that it is a issue of interpretation of results from different techniques. In the original paper Hernanz-Falcon *et al.*, (2004) utilised a single cell FRET approach that the group claim is much more sensitive in comparison to BRET. However, the group also states that energy transfer could be observed for both wild type and mutant forms of CCR5 at high levels of receptor expression while at medium expression levels FRET was observed only for the wild-type receptors. It is clear that the mechanisms involved in receptor oligomerisation are complex and further studies are required to accurately resolve if there is a general mechanism for oligomer formation across the chemokine receptor family or if each receptor has an individual mechanism for oligomerisation. The latter possibility is somewhat unsatisfying given the large degree of sequence homology observed among the chemokine receptor family.

Figure 4.1 Co-immunoprecipitation of differentially epitope-tagged forms of the human CXCR1 receptor.

HEK293 cells were transfected to transiently express Flag-CXCR1 (lane 2) or c-Myc-CXCR1 (lane 3). Flag-CXCR1 and c-Myc-CXCR1 were coexpressed (lane 4). A non-transfected HEK293T (lane 1) and a mixed cell control (lane 5) were included. The mixed cell control represents cells individually expressing the constructs and mixed prior to immunoprecipitation. Cell lysates were immunoprecipitated with anti-Flag antibody, samples resolved by SDS-PAGE and then immunoblotted with anti-c-Myc antibody (upper panel). Western blot analysis of cell lysates using anti-Flag and anti-c-Myc antibodies was also performed in order to ensure correct protein expression (lower panels). One experiment representative of three experiments is shown.

Figure 4.1

Mr ($\times 10^{-3}$)

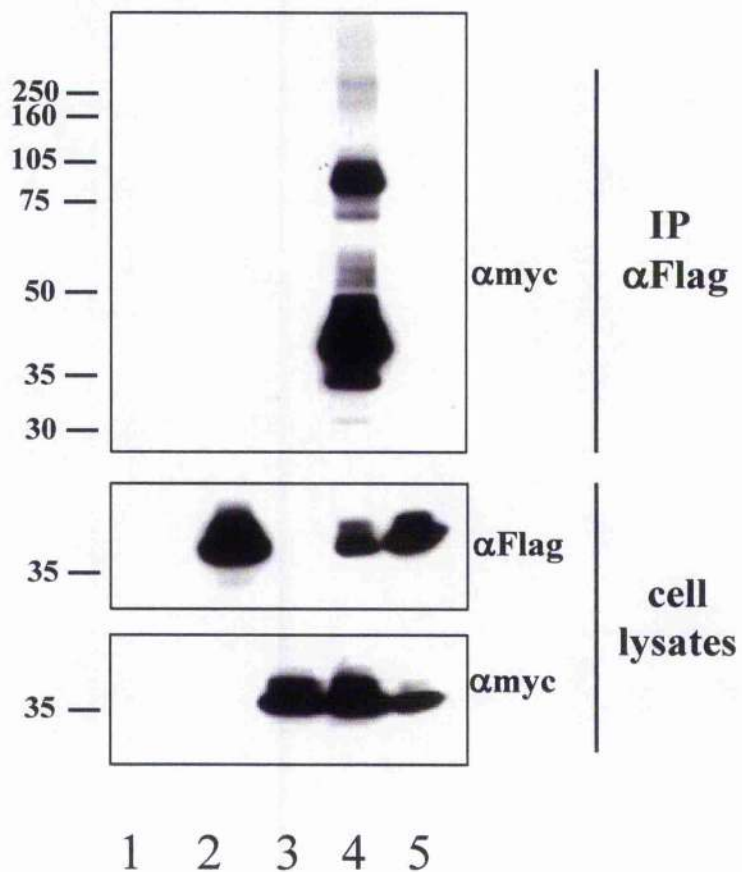


Figure 4.2 Co-immunoprecipitation of differentially epitope-tagged forms of the human CXCR2 receptor

HEK293 cells were transfected to transiently express Flag-CXCR2 (lane 2) or c-Myc-CXCR2 (lane 3). Flag-CXCR2 and c-Myc-CXCR2 were coexpressed (lane 4). A non-transfected HEK293T (lane 1) and a mixed cell control (lane 5) were included. The mixed cell control represents cells individually expressing the constructs and mixed prior to immunoprecipitation. Cell lysates were immunoprecipitated with anti-Flag antibody, samples resolved by SDS-PAGE and then immunoblotted with anti-c-Myc antibody (upper panel). Western blot analysis of cell lysates using anti-Flag and anti-c-Myc antibodies was also performed in order to ensure correct protein expression (lower panels). One experiment representative of three experiments is shown.

Figure 4.2

Mr ($\times 10^{-3}$)

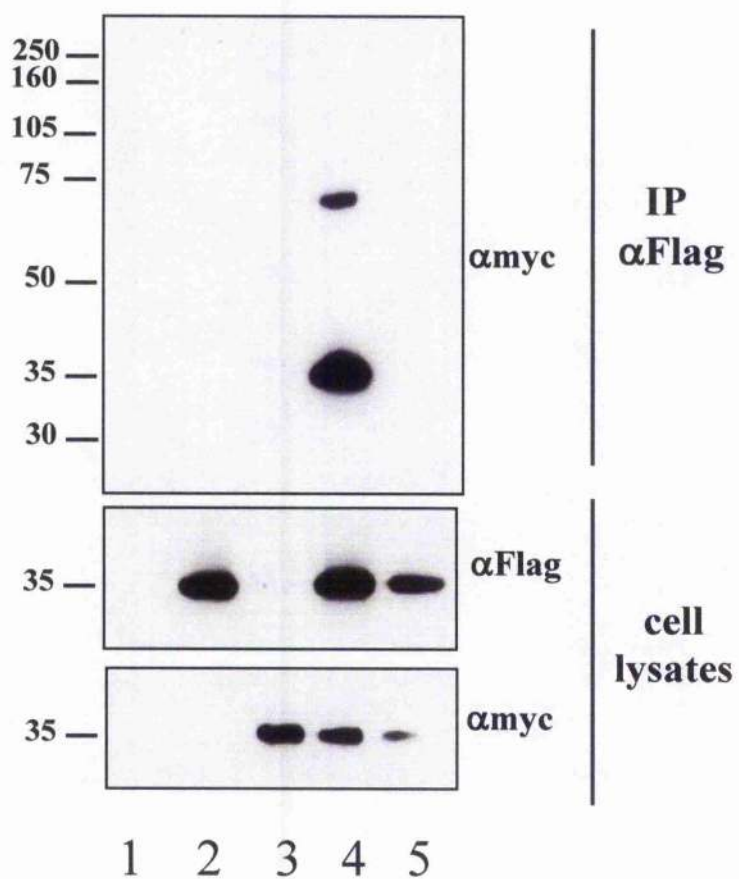


Figure 4.3 Co-immunoprecipitation of differentially epitope-tagged forms of the human CXCR1 and CXCR2 receptor

HEK293 cells were transfected to transiently express Flag-CXCR1 (lane 2) or c-Myc-CXCR2 (lane 3). Flag-CXCR1 and c-Myc-CXCR2 were coexpressed (lane 4). A non-transfected HEK293T (lane 1) and a mixed cell control (lane 5) were included. The mixed cell control represents cells individually expressing the constructs and mixed prior to immunoprecipitation. Cell lysates were immunoprecipitated with anti-Flag antibody, samples resolved by SDS-PAGE and then immunoblotted with anti-c-Myc antibody (upper panel). Western blot analysis of cell lysates using anti-Flag and anti-c-Myc antibodies was also performed in order to ensure correct protein expression (lower panels). One experiment representative of three experiments is shown.

Figure 4.3

Mr ($\times 10^{-3}$)

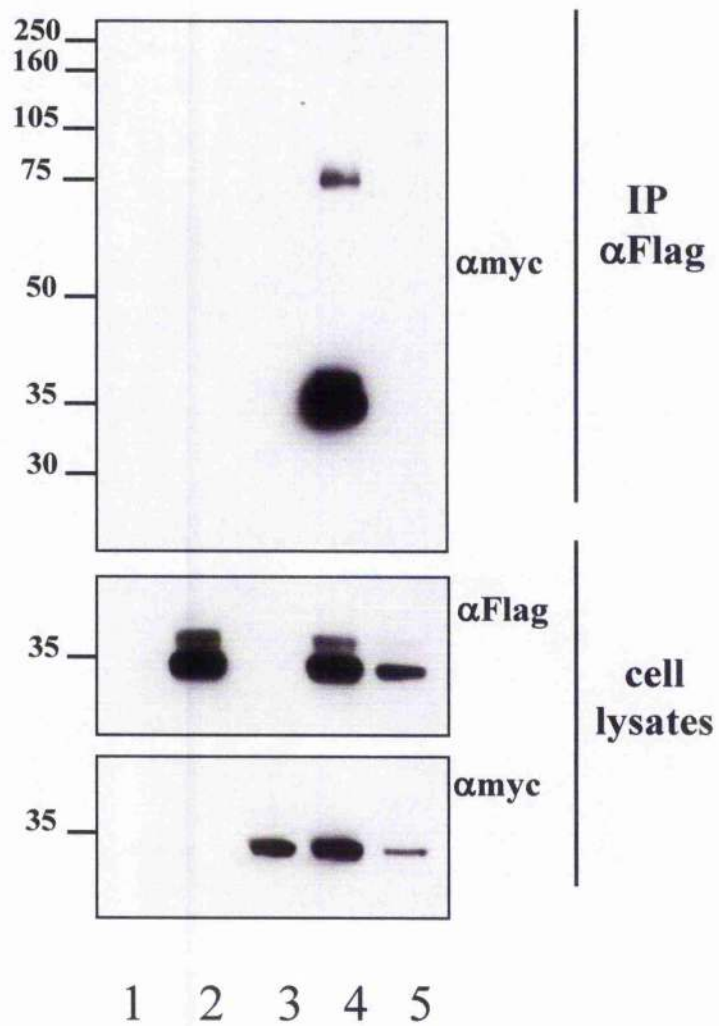
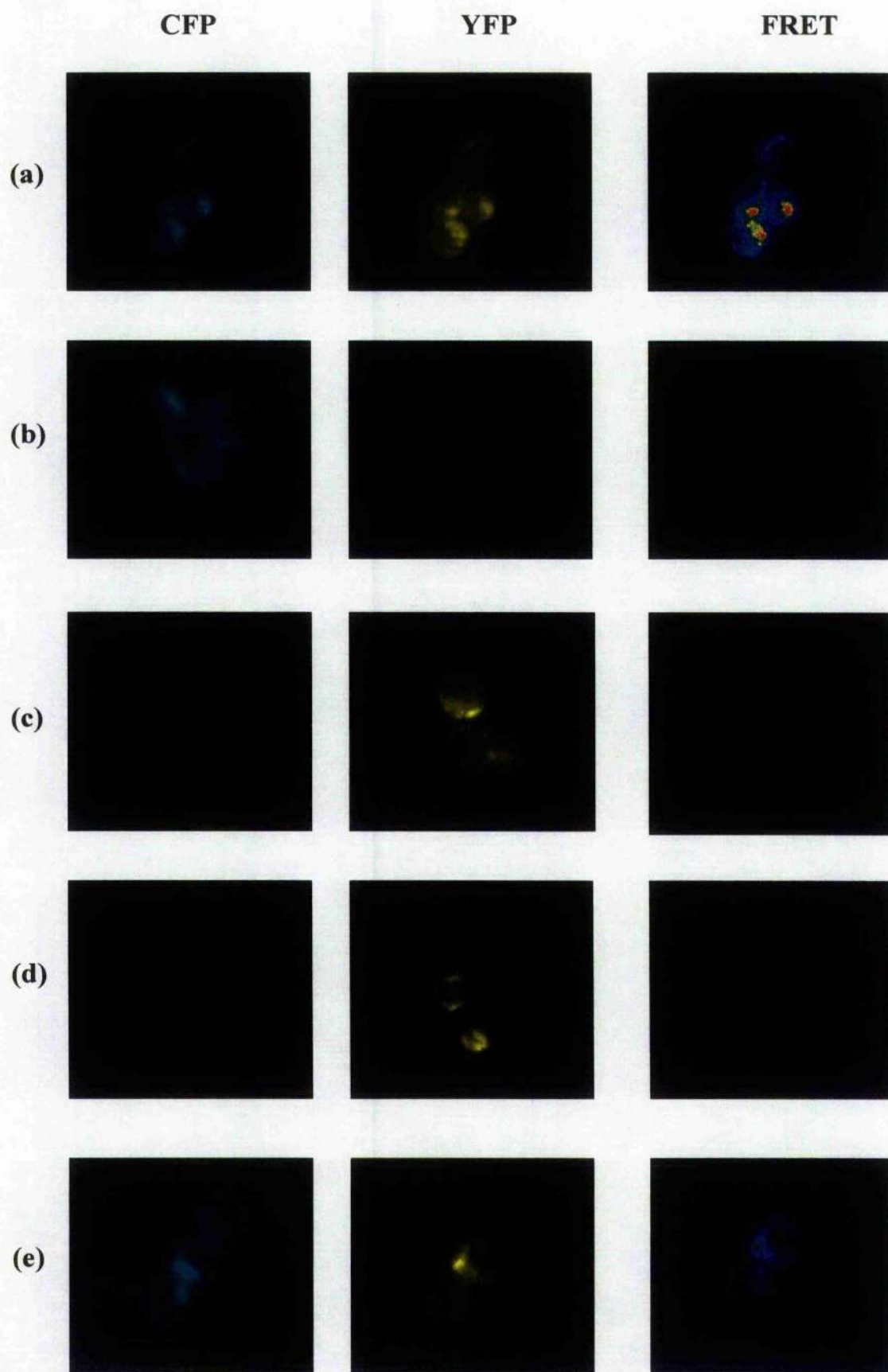


Figure 4.4 FRET imaging of constitutive CXCR1 homo-oligomerisation in single cells

CXCR1CFP and CXCR1YFP were co-expressed (a) or expressed individually (CXCR1CFP- b, CXCR1YFP - c) in HEK293T cells. As a control interactions with the α_{1A} -adrenoceptor were recorded. α_{1A} -adrenoceptor-YFP was expressed alone (d) or co-expressed with CXCR1CFP (e). Left hand panels represent CFP images, centre panels represent YFP images and right hand panels represent corrected FRET. FRET signals ($\text{FRET}_{\text{NORM}}$) were quantified as described in section 2.6.9, shown in (f). Data shown are mean \pm S.E.M. from three experiments.

Figure 4.4



(f)

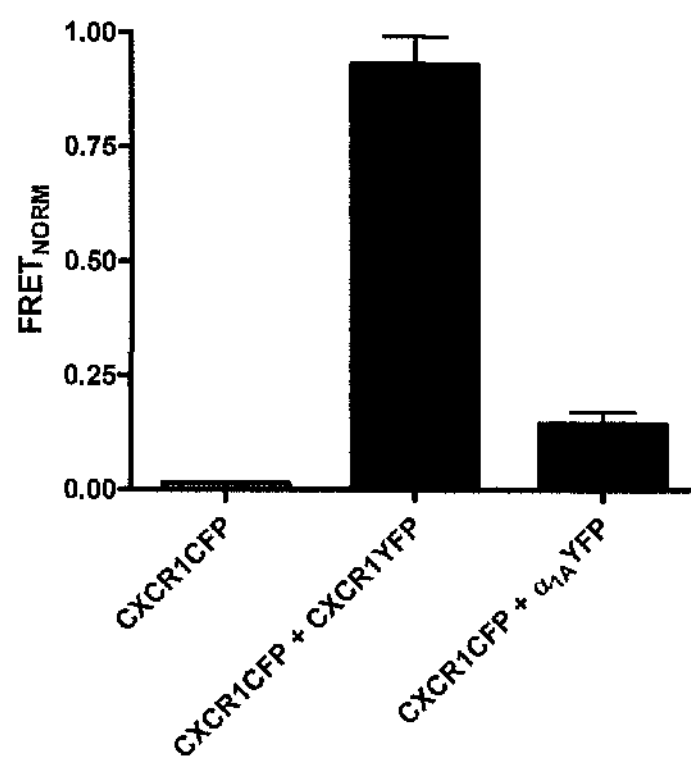
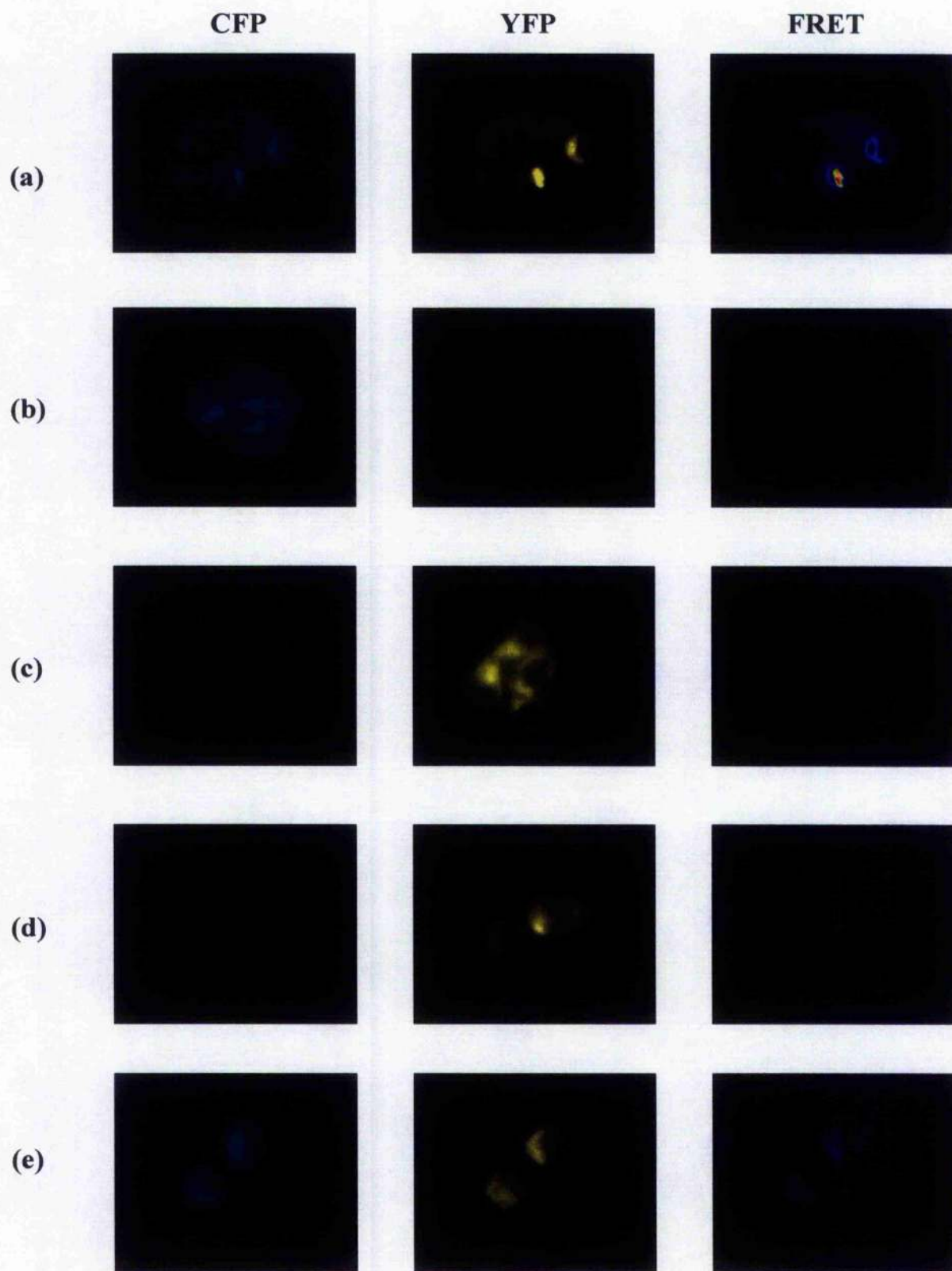


Figure 4.5 FRET imaging of constitutive CXCR2 homo-oligomerisation in single cells

CXCR2CFP and CXCR2YFP were co-expressed (a) or expressed individually (CXCR2CFP- b, CXCR2YFP - c) in HEK293T cells. As a control interactions with the α_{1A} -adrenoceptor were recorded. α_{1A} -adrenoceptor-YFP was expressed alone (d) or co-expressed with CXCR2CFP (e). Left hand panels represent CFP images, centre panels represent YFP images and right hand panels represent corrected FRET. FRET signals ($\text{FRET}_{\text{NORM}}$) were quantified as described in section 2.6.9, shown in (f). Data shown are mean \pm S.E.M. from three experiments.

Figure 4.5



(f)

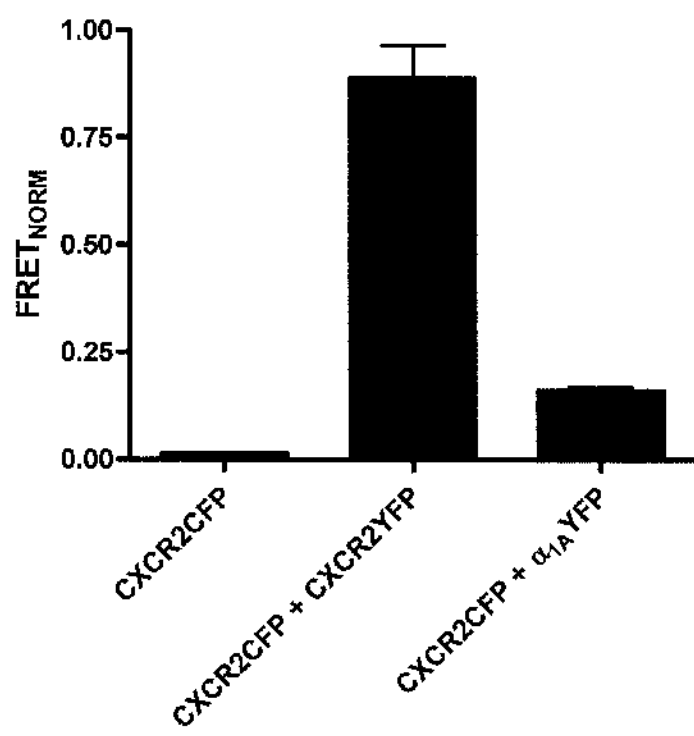
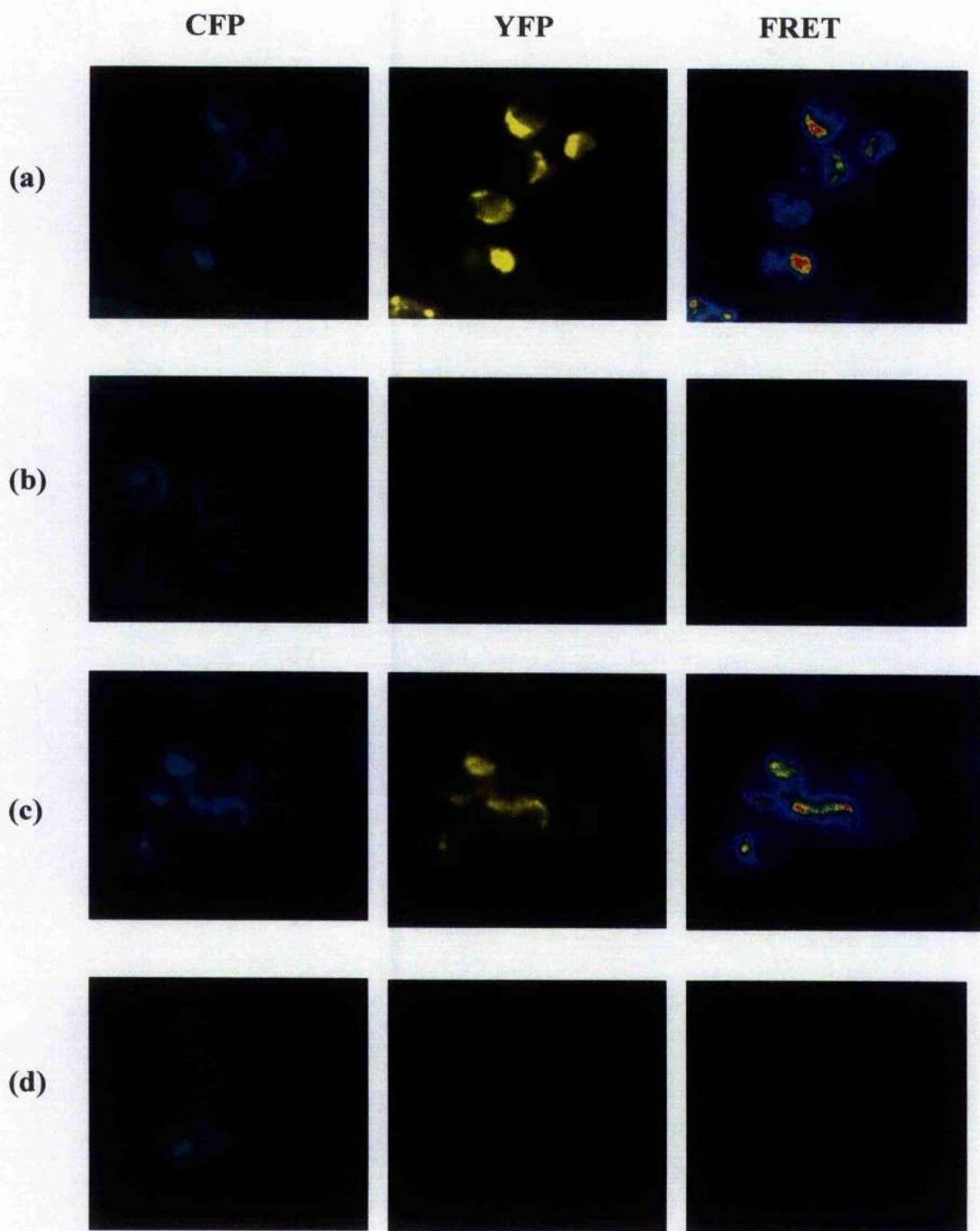


Figure 4.6 FRET imaging of constitutive CXCR1/CXCR2 hetero-oligomerisation in single cells

CXCR1CFP was co-expressed with CXCR2YFP (a) or expressed alone (b) in HEK293T cells. The opposite pairing of CXCR2CFP and CXCR1YFP were co-expressed (c) and CXCR2CFP was expressed alone (d). Left hand panels represent CFP images, centre panels represent YFP images and right hand panels represent corrected FRET. FRET signals ($\text{FRET}_{\text{NORM}}$) were quantified as described in section 2.6.9, shown in (f). Data shown are mean \pm S.E.M. from three experiments.

Figure 4.6



(e)

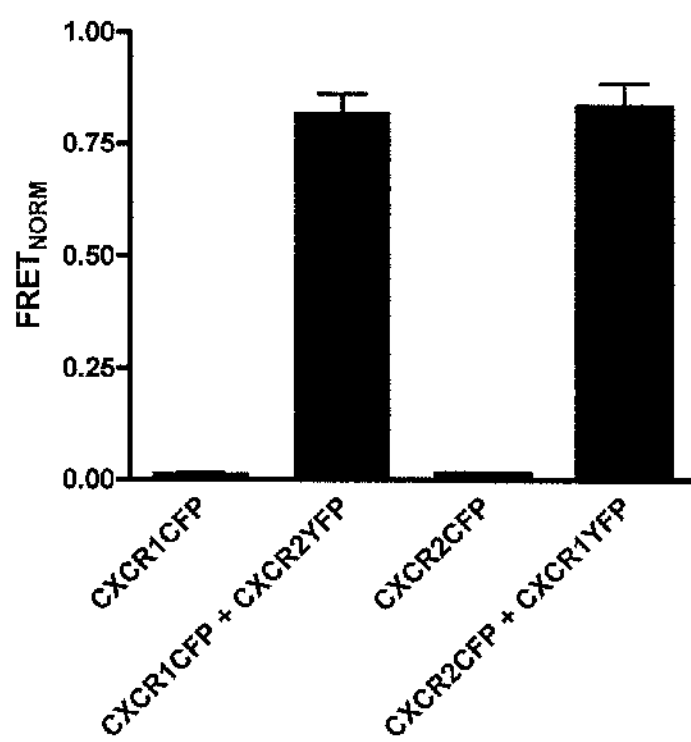
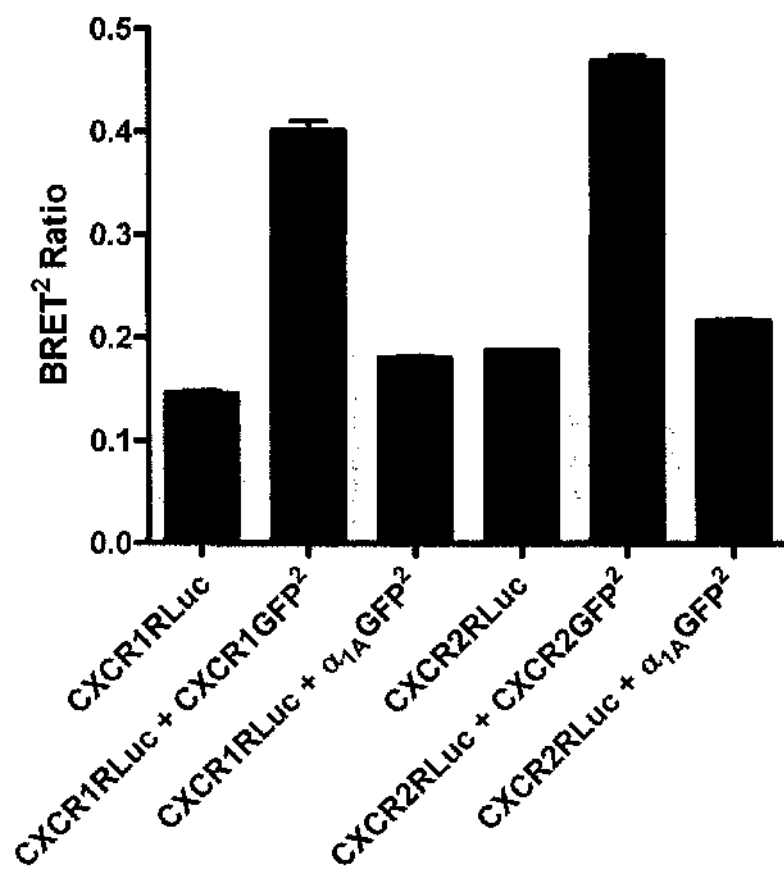


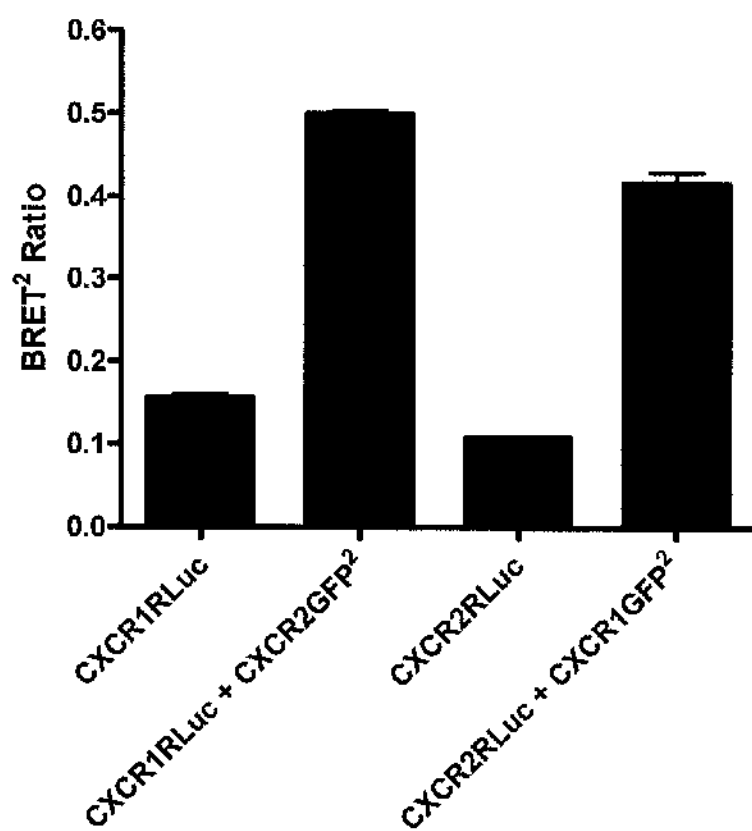
Figure 4.7 Single point BRET² analysis of CXCR1 and CXCR2 homo- and hetero-oligomers

BRET² ratio was measured in intact cells transfected with equal amounts of cDNA corresponding to donor and acceptor. Donor was expressed alone or co-expressed with acceptor for CXCR1 and CXCR2 homo-oligomer pairs (a). CXCR1Rluc and CXCR2Rluc were also co-expressed with α_{1A} -adrenoceptor-GFP² and energy transfer recorded. BRET² signals resulting from CXCR1/CXCR2 hetero-oligomer formation are also shown (b). Data are means \pm S.E.M. from three experiments.

Figure 4.7



(a)



(b)

Figure 4.8 BRET² saturation analysis of CXCR1 and CXCR2 homo- and hetero-oligomerisation

Homo-oligomerisation of CXCR1 (inverted triangles) and CXCR2 (diamonds) was investigated using saturation BRET². Varying amounts of receptor-Rluciferase conjugate (donor) and receptor-GFP² conjugate (acceptor) were expressed in HEK293T cells and BRET recorded following addition of DeepBlueC substrate. Hetero-oligomer formation between CXCR1 and CXCR2 was also assessed (squares). The specificity of CXCR2 receptor interactions was investigated following co-expression of CXCR2Rluc with α_{1A} -adrenoceptor-GFP² (circles). A lack of inherent interaction between Rluc and GFP² was demonstrated by co-expression of CXCR1Rluc and GFP² (triangles).

Figure 4.8

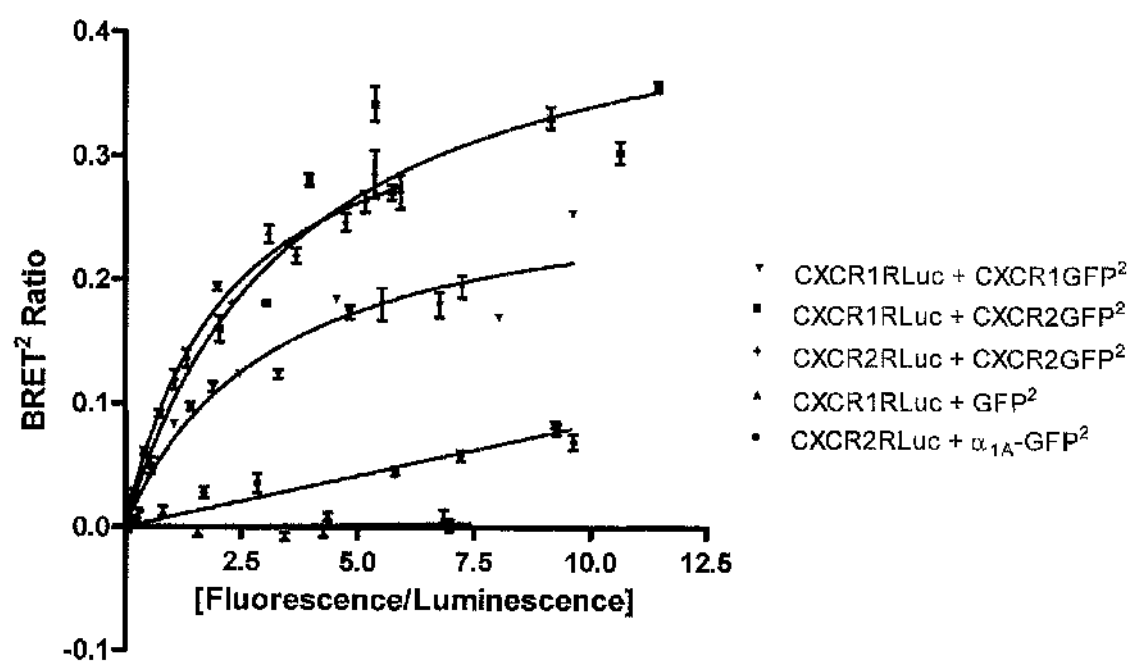
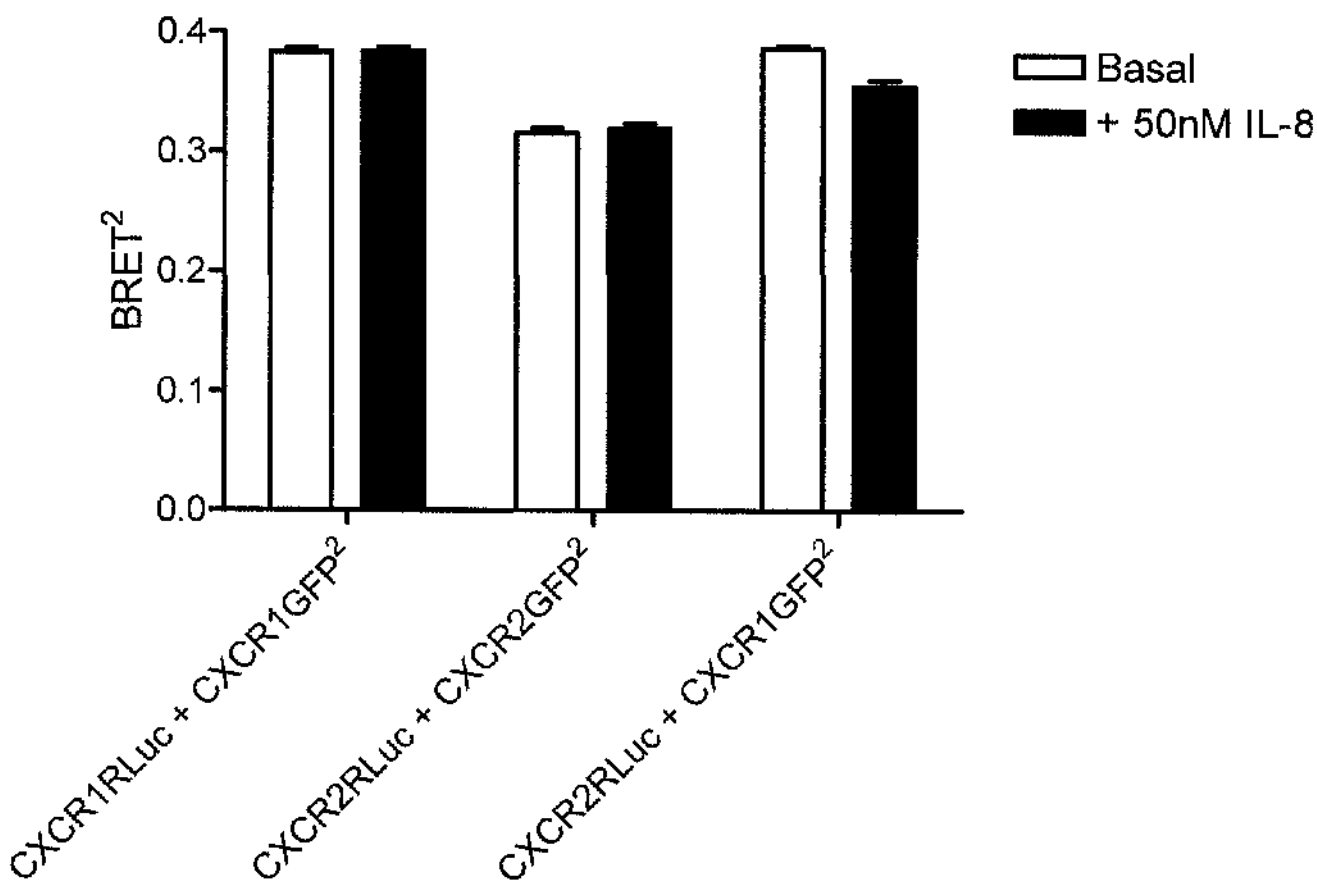


Figure 4.9 IL-8 incubation does not modulate BRET² resulting from CXCR1 and CXCR2 homo- and hetero-oligomeric interactions

BRET² ratio was measured in intact HEK293T cells expressing equal ratios of CXCR1Rluc and CXCR1GFP², CXCR2Rluc and CXCR2GFP² and CXCR2Rluc and CXCR1GFP². Cells were incubated for 30 minutes with 50nM IL-8 and BRET² measured.

Graph shown is representative of results obtained for three individual experiments.

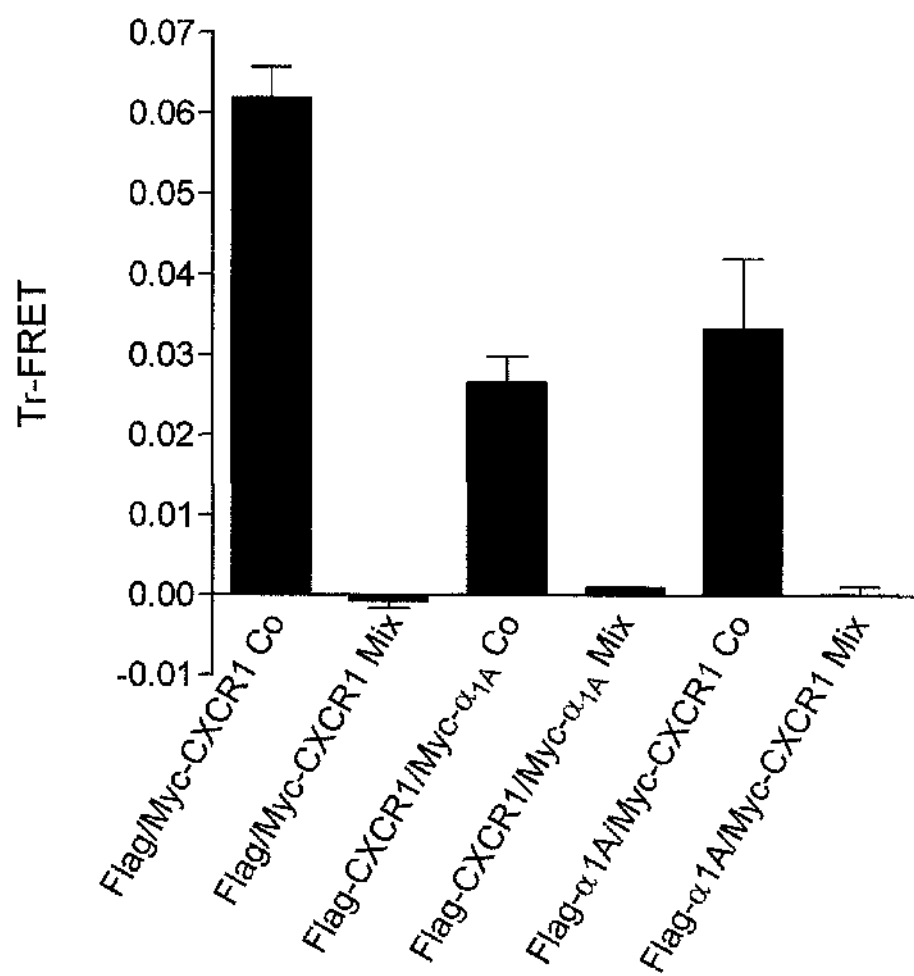
Figure 4.9



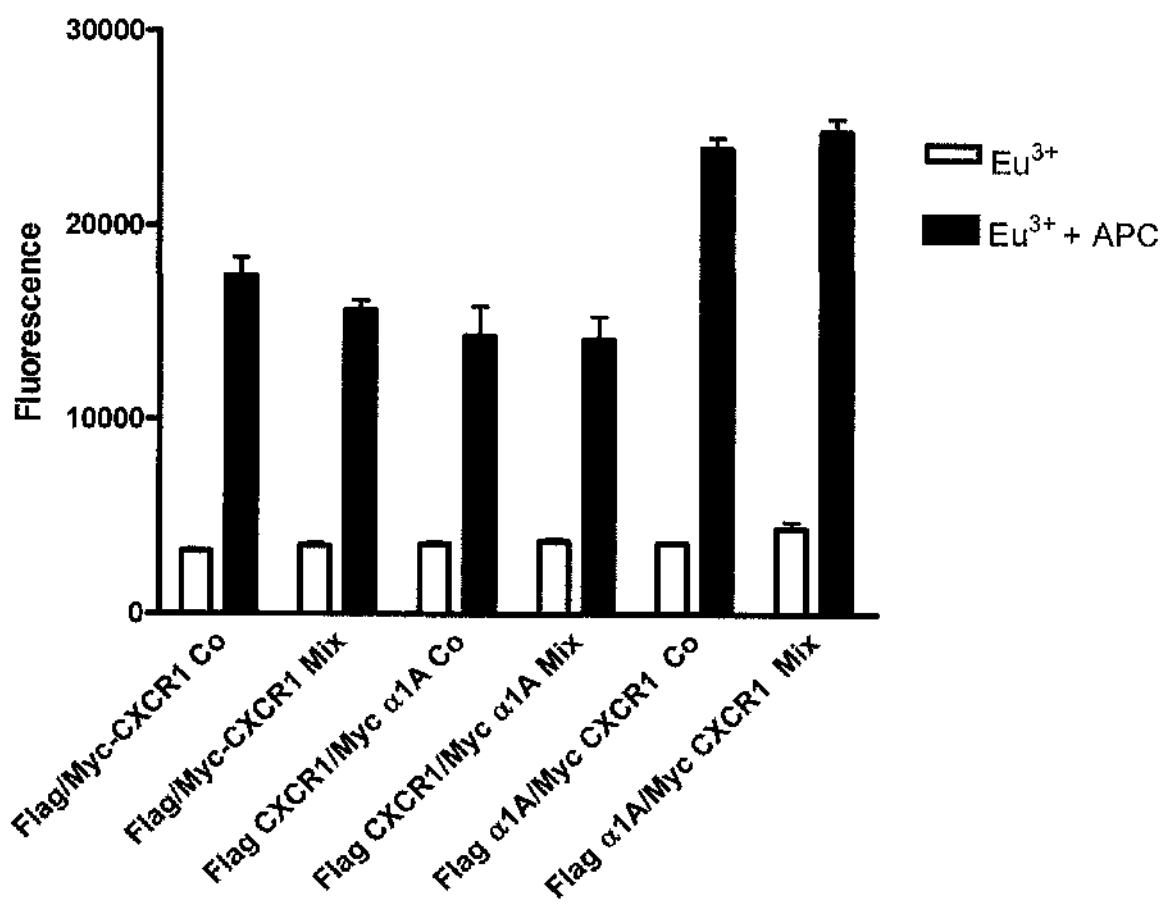
**Figure 4.10 Constitutive CXCR1 homo-oligomer formation at the cell surface
detected using Tr-FRET**

HEK293T cells were transiently transfected with N-terminally tagged Flag-CXCR1 and c-Myc-CXCR1 either together (co) or individually and mixed prior to antibody incubation (mix). To investigate the specificity of energy transfer N-terminally Flag or c-Myc- α_{1A} -adrenoceptor was coexpressed with Flag- or c-Myc-CXCR1. Data represent means \pm S.E.M. from three independent experiments. To ensure correct expression of the Flag-tagged receptor acting as an energy acceptor, APC fluorescence was measured at 665nm (b). Data shown are representative of three individual experiments.

Figure 4.10



(a)

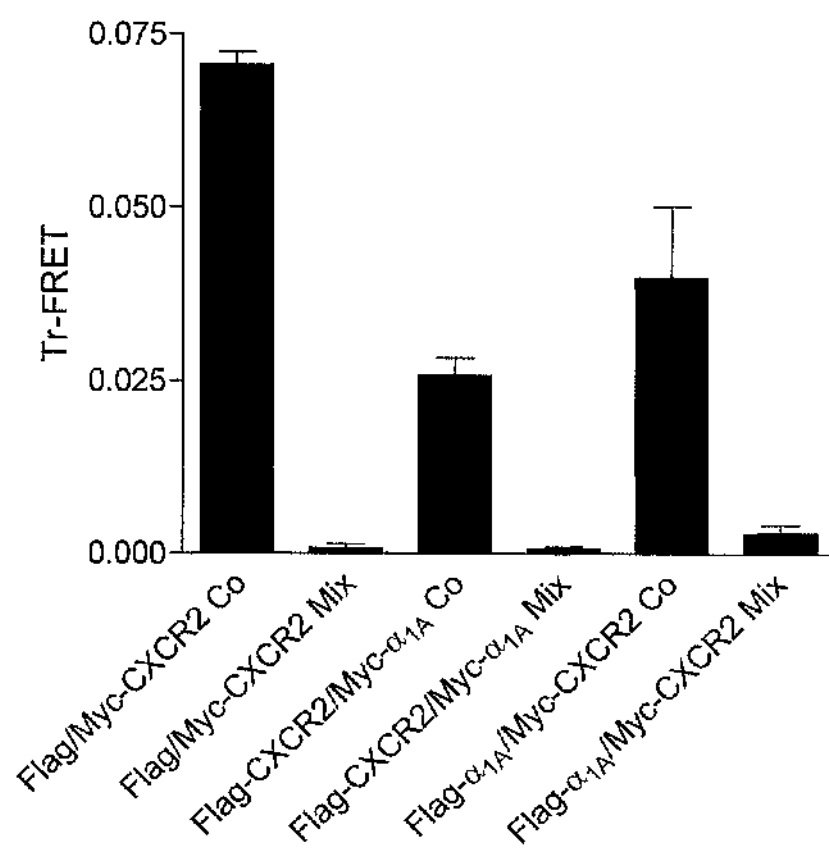


(b)

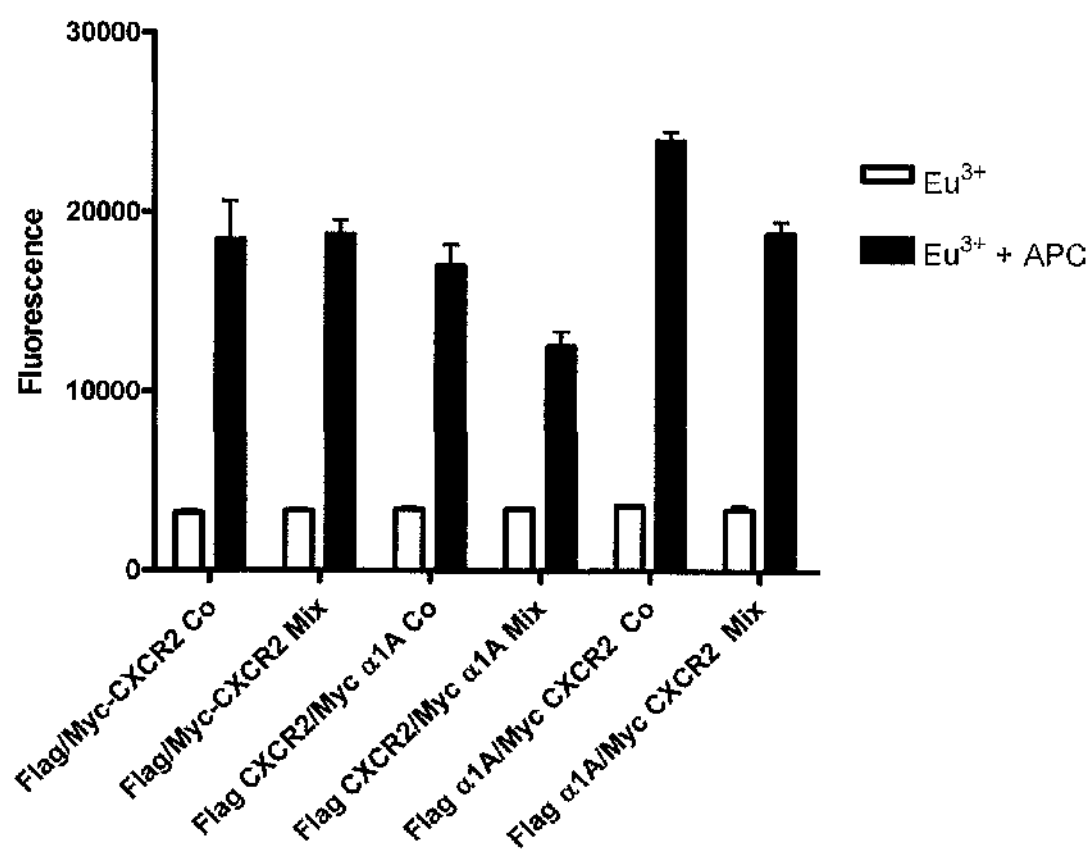
**Figure 4.11 Constitutive CXCR2 homo-oligomer formation at the cell surface
detected using Tr-FRET**

HEK293T cells were transiently transfected with N-terminally tagged Flag-CXCR2 and c-Myc-CXCR2 either together (co) or individually and mixed prior to antibody incubation (mix). To investigate the specificity of energy transfer N-terminally Flag or c-Myc- α_{1A} -adrenoceptor was coexpressed with Flag- or c-Myc-CXCR2. Data represent means \pm S.E.M. from three independent experiments. To ensure correct expression of the Flag-tagged receptor acting as an energy acceptor, fluorescence was measured at 665nm (b). Data shown are representative of three individual experiments.

Figure 4.11



(a)

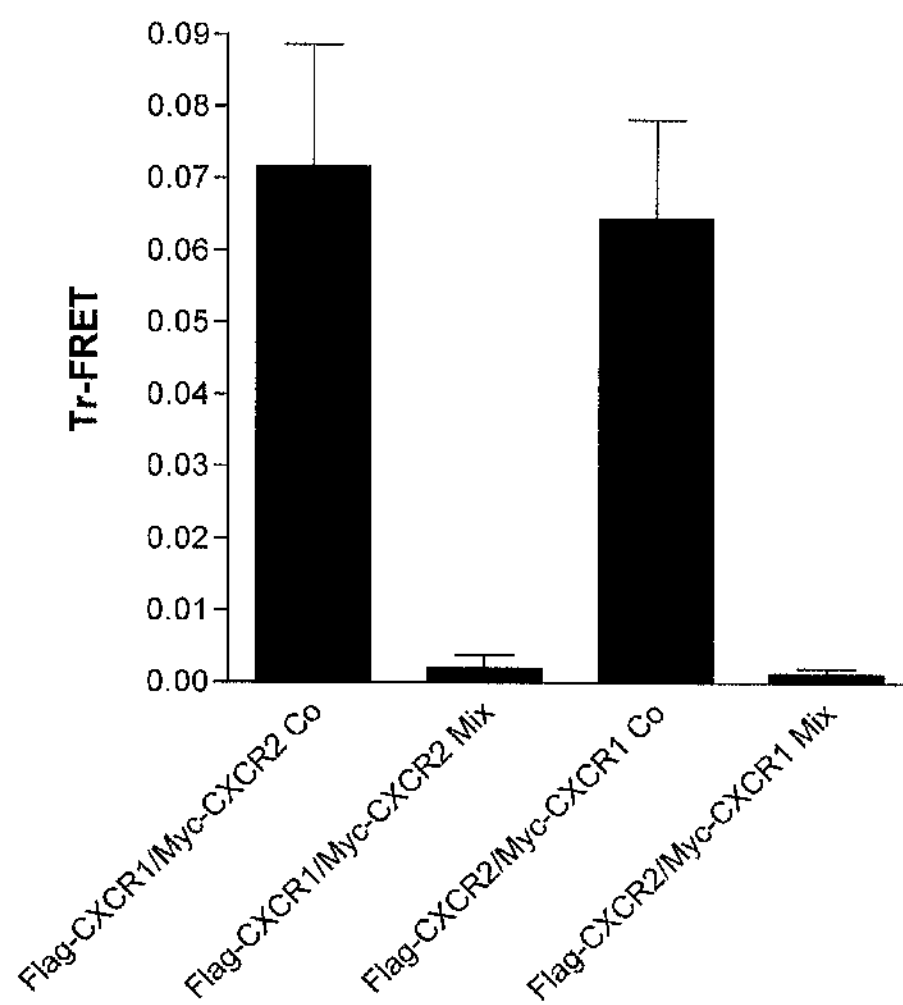


(b)

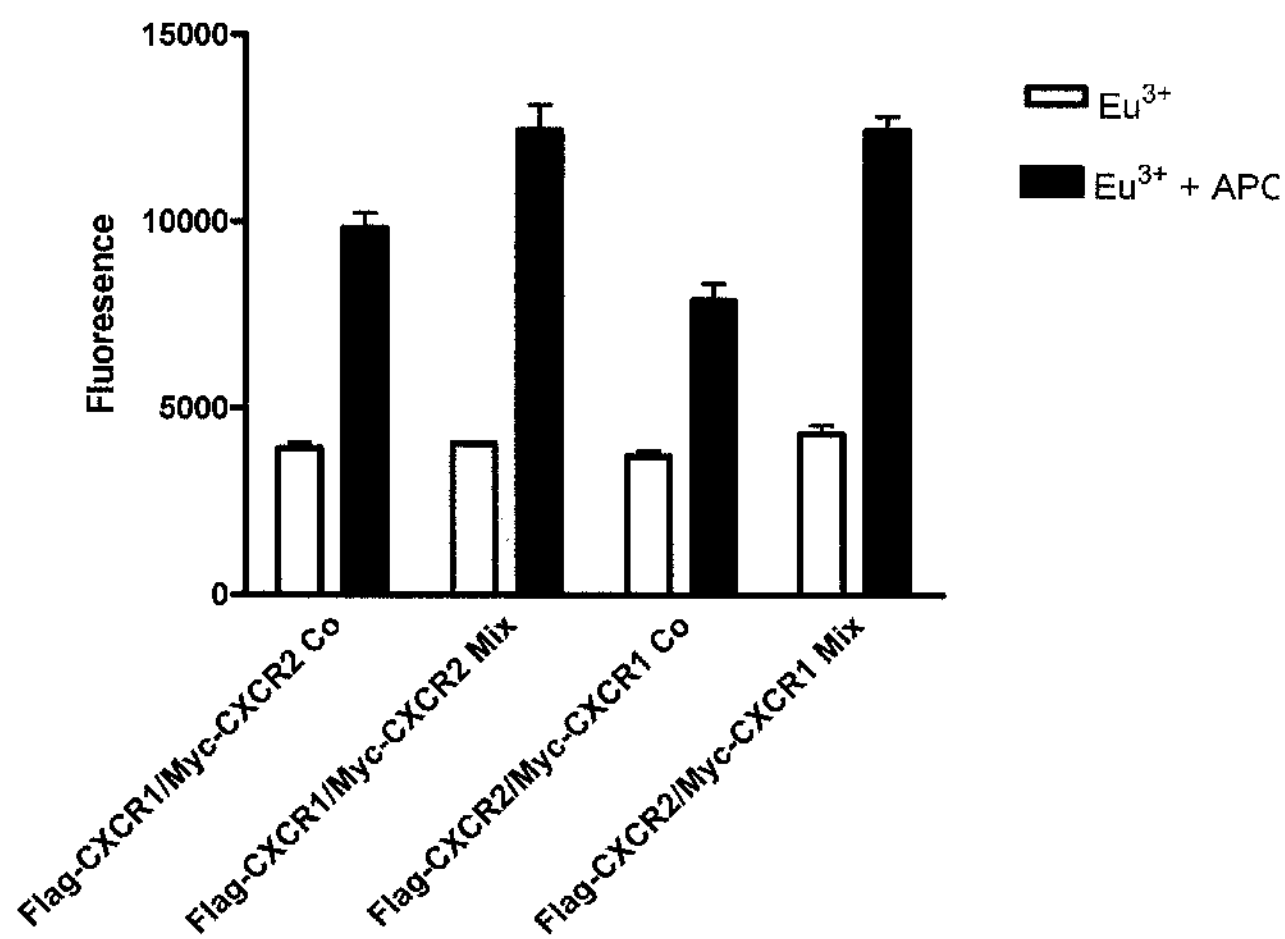
**Figure 4.12 Constitutive CXCR1/2 hetero-oligomer formation at the cell surface
detected using Tr-FRET**

HEK293T cells were transiently transfected with N-terminally tagged Flag-CXCR2 and c-Myc-CXCR1 either together (co) or individually and mixed prior to antibody incubation (mix). The opposite pairing of Flag-CXCR1 and c-Myc-CXCR2 was also tested. Data represent means \pm S.E.M. from three independent experiments. To ensure correct expression of the Flag-tagged receptor acting as an energy acceptor, fluorescence was measured at 665nm (b). Data shown are representative of three individual experiments.

Figure 4.12



(a)



(b)

Figure 4.13 CXCR1 and CXCR2 homo- and hetero-oligomerisation at the cell surface monitored by Tr-FRET is not modulated by incubation with IL-8

HEK293T cells were transiently transfected with combinations of N-terminally tagged Flag-CXCR1/2 and c-Myc-CXCR1/2 either together (co) or individually and mixed prior to antibody incubation (mix). Cells were treated with vehicle (open bars) or 100nM IL-8 (closed bars). Data represent means \pm S.E.M. from three independent experiments.

Figure 4.13

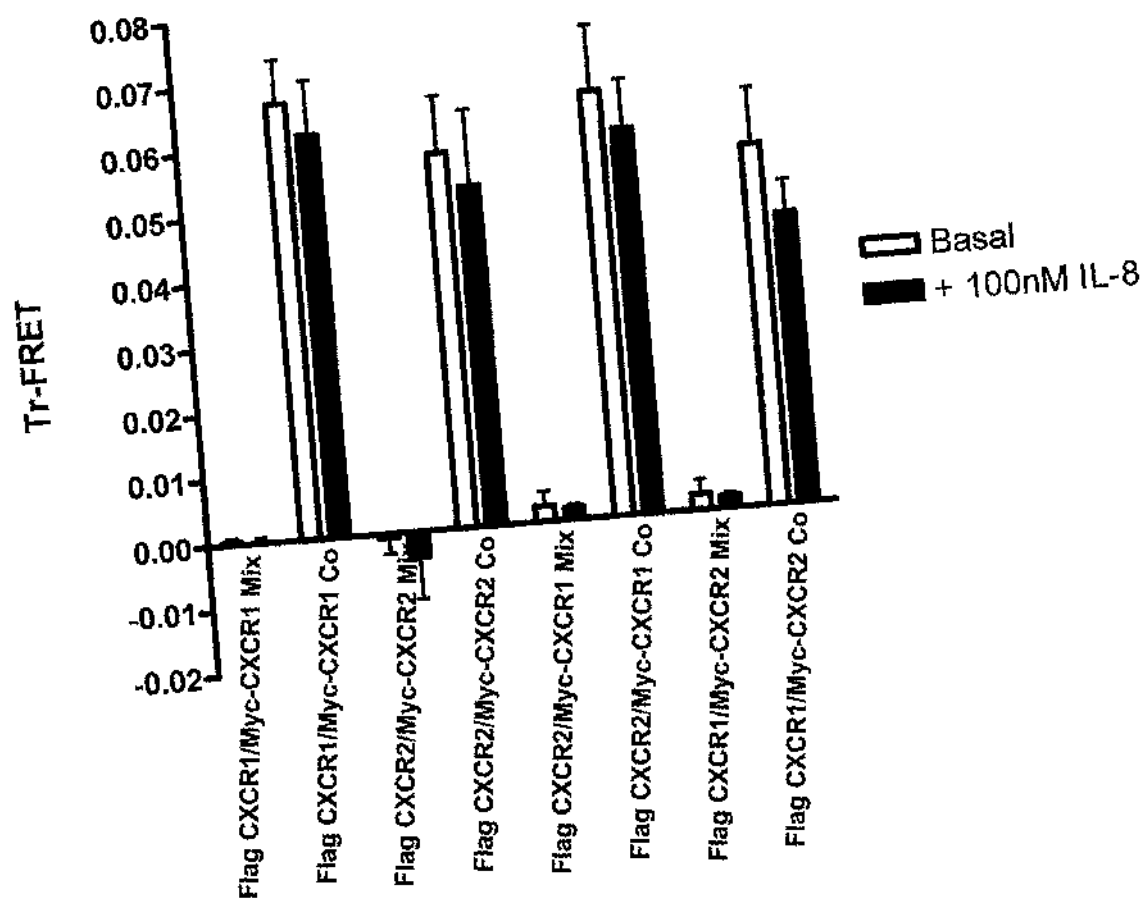


Figure 4.14 Transient expression of CXCR1, HA- α_{2C} -adrenoceptor and HA-CXCR1-ER constructs in HEK293T cells

HEK293T cells were transiently transfected with CXCR1 (a), HA- α_{2C} -adrenoceptor (b) and HA-CXCR1-ER (c). 24 hours post-transfection immunocytochemistry was performed. Images are shown for non-permeabilised samples (left hand panel) and permeabilised samples (right hand panel). Images shown are representative of three individual experiments.

Figure 4.14

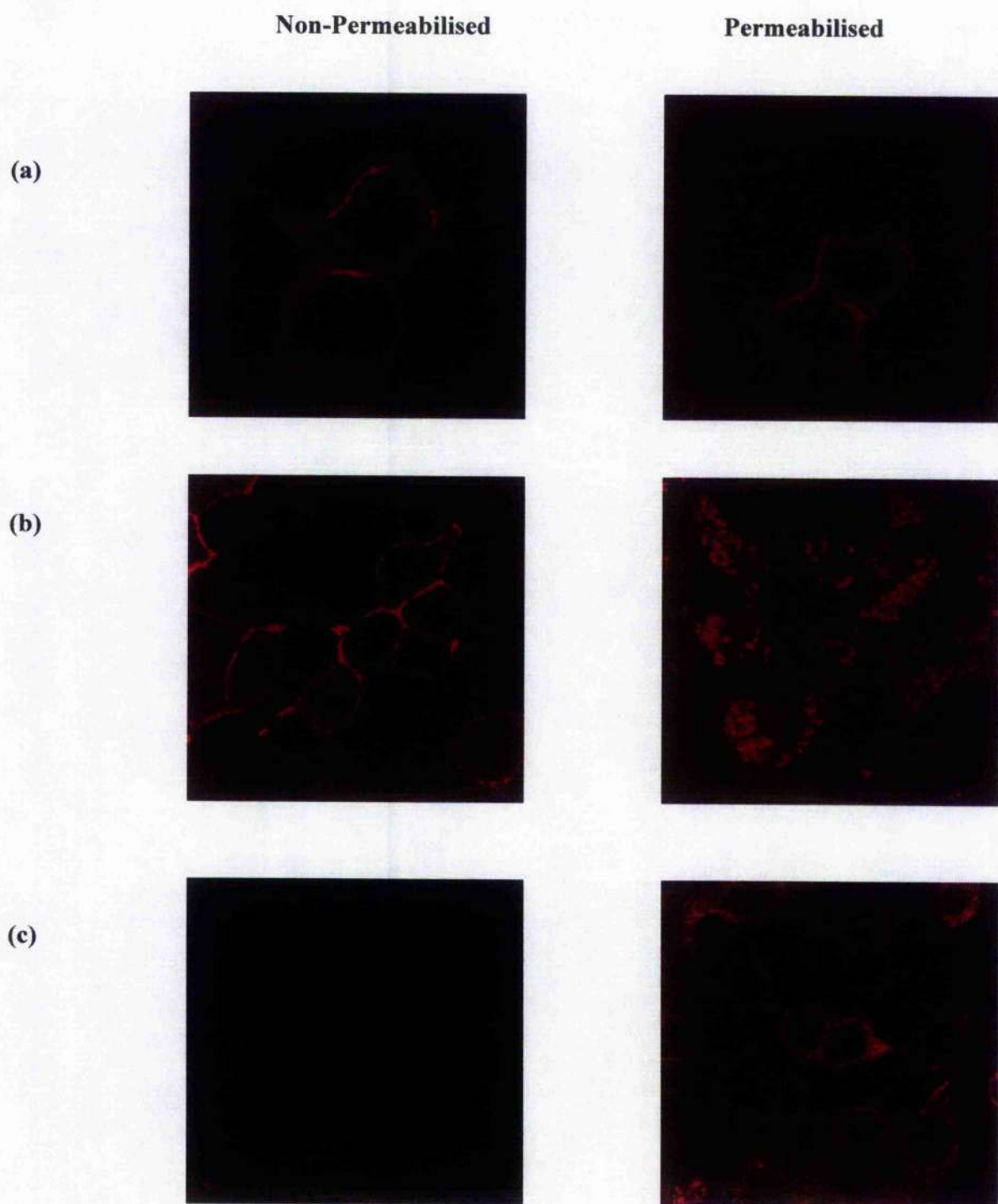


Figure 4.15 Overlapping expression patterns of HA-CXCR1-ER and c-Myc-CXCR1 and CXCR2.

HEK293T cells were transiently transfected with HA-CXCR1-ER and c-Myc-CXCR1 (a) or c-Myc-CXCR2 (b). 24 hours post-transfection immunocytochemistry was performed. Cells were permeabilised prior to staining with anti-HA antibody (green) and anti-c-Myc antibody (red). Left hand panel represents anti-HA staining, centre panel represents anti-c-Myc staining and the right hand panel represents the image overlay. Images shown are representative of three individual experiments.

Figure 4.15

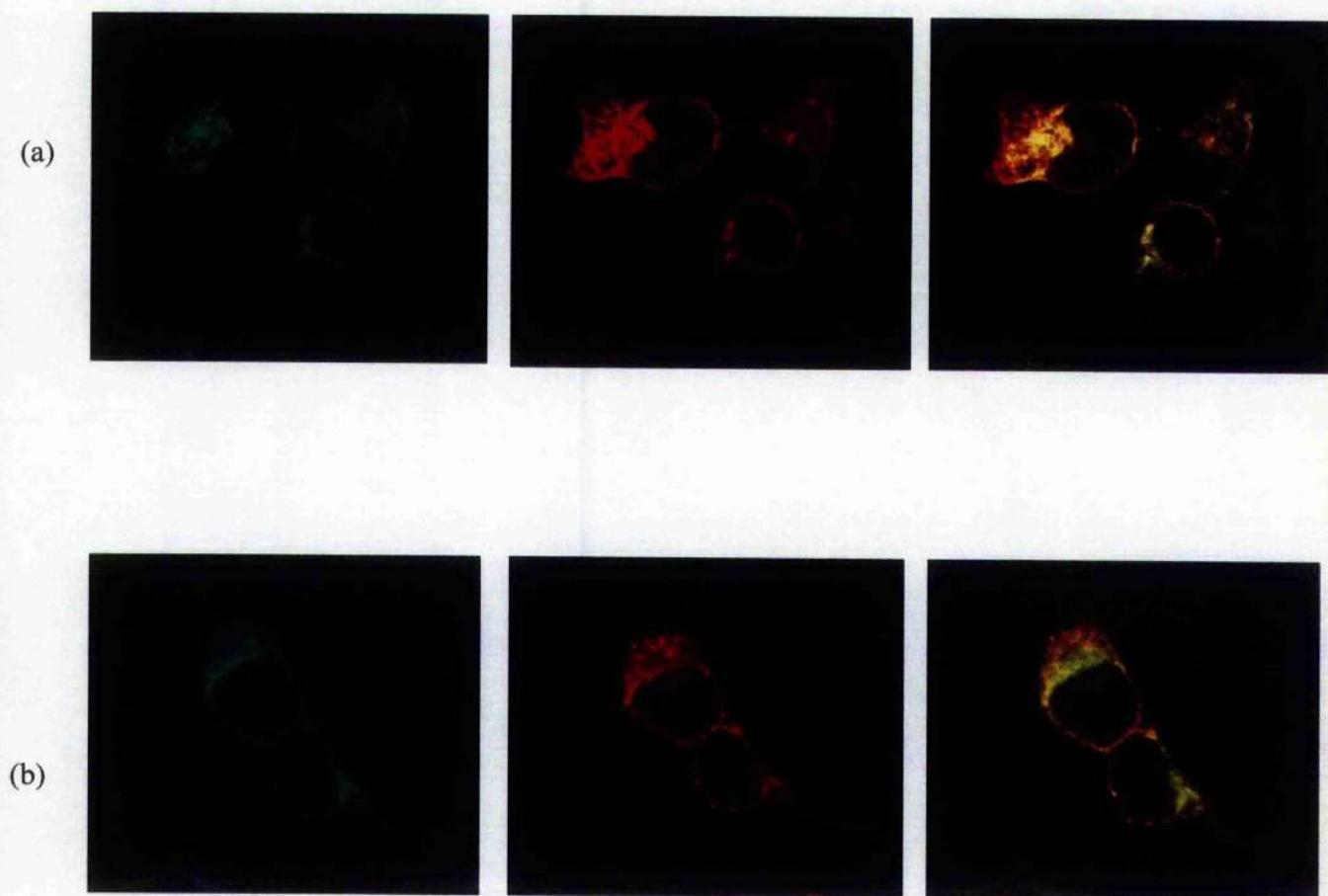


Figure 4.16 HA-CXCR1-ER intracellularly retains CXCR1 and CXCR2 but not the α_{1A} -adrenoceptor

HEK293T cells were transfected with either HA-CXCR1 (open bars) or HA-CXCR1-ER (closed bars) along with Flag-CXCR1, Flag-CXCR2 or Flag- α_{1A} -adrenoceptor. An equal amount of cells were incubated with anti-Flag antibody conjugated to APC for 2 hours prior to washing. APC fluorescence was then quantified and background fluorescence of HEK293T was measured and removed from the values obtained. Data are means \pm S.E.M. from three individual experiments. Data were analysed using Students unpaired T-test (GraphPad prism 4). ***, $p < 0.001$.

Figure 4.16

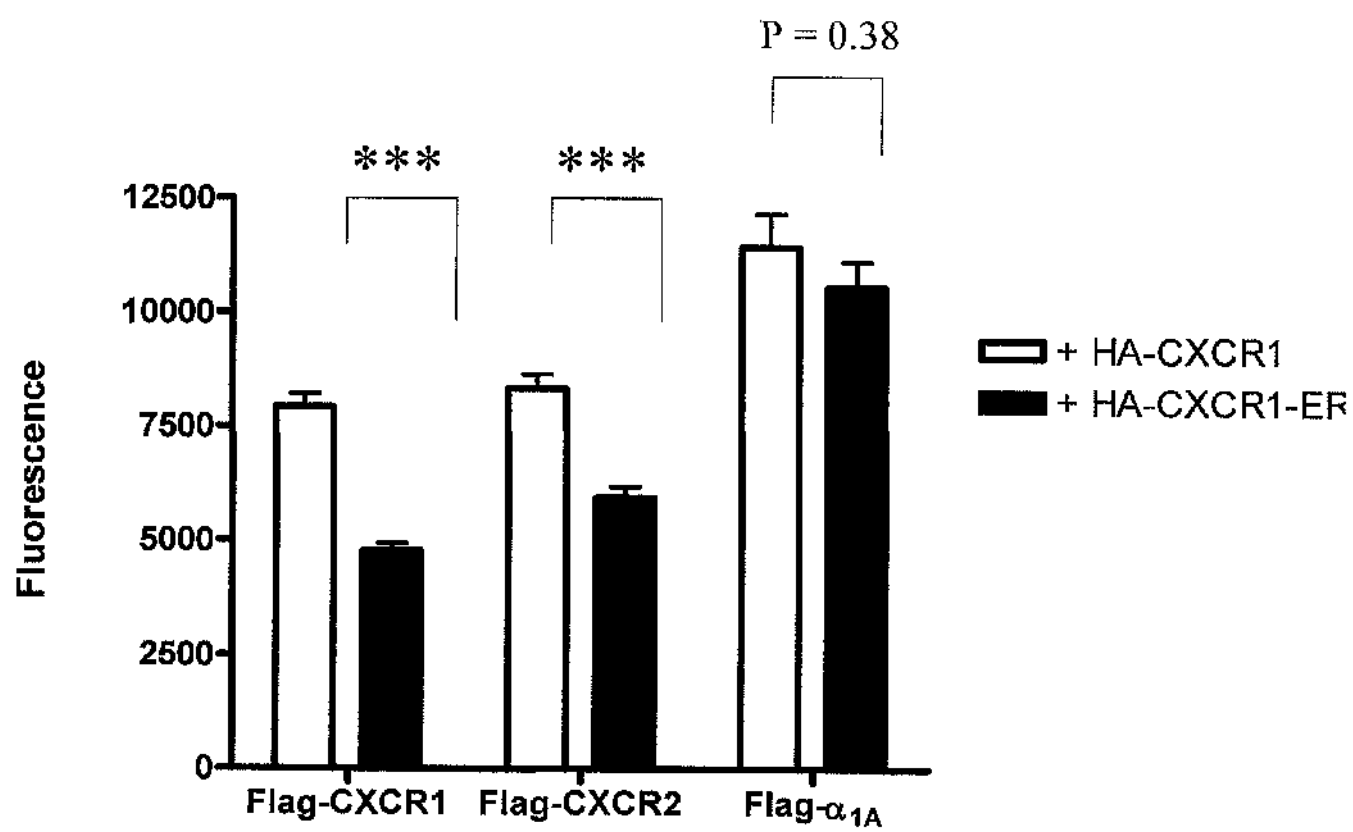
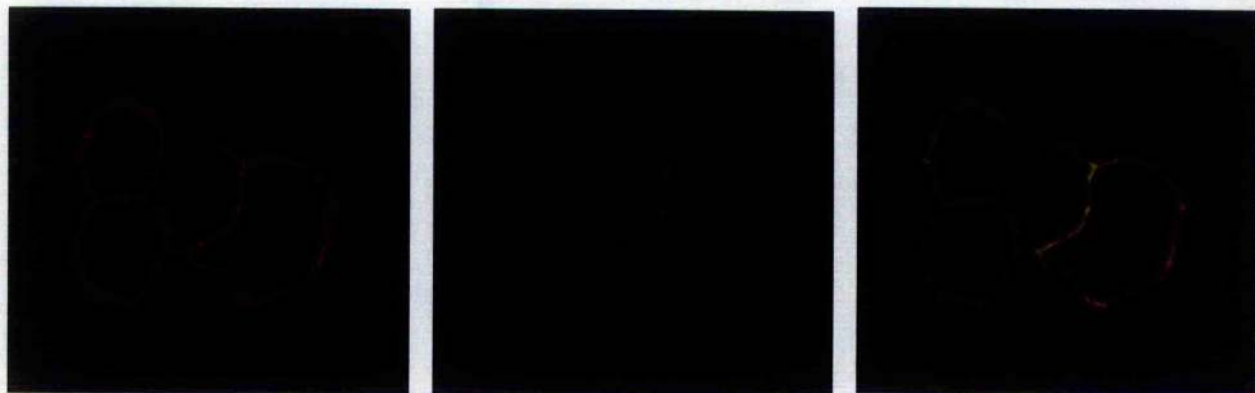


Figure 4.17 Lack of co-internalisation of CXCR1 and CXCR2

c-Myc-CXCR1 and VSV-CXCR2 were transiently co-expressed in HEK293T cells grown on coverslips and immunocytochemistry performed using anti-c-Myc (red) and anti-VSV (green) antibodies. Cells were stained prior to agonist stimulation (a), following exposure to a CXCR1 and CXCR2 agonist IL-8 (50nM) for 30 minutes (b) and following exposure to a CXCR2 selective agonist GRO- α (100nM) for 30 minutes (c). Left hand panel represents anti-c-Myc staining, centre panel represents anti-VSV staining and the right hand panel represents the image overlay. Images shown are representative of three independent experiments.

Figure 4.17

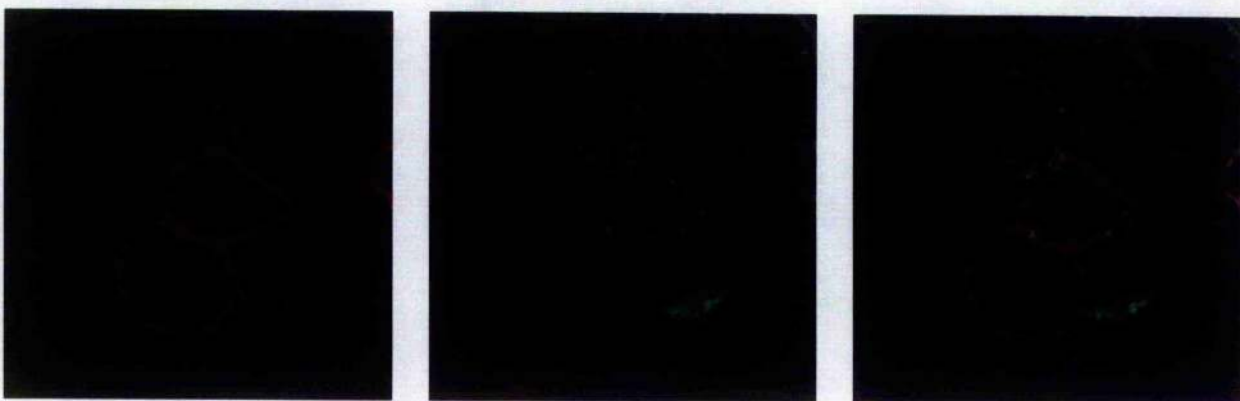
(a)



(b)



(c)



Chapter Five

5.1 Introduction

Opioid receptors are members of the class A GPCR family. Pharmacological binding and anatomical distribution analyses have identified three opioid family members μ (MOP), δ (DOP) and κ (KOP) receptors expressed in human brain tissue (Chen *et al.*, 1993; Li *et al.*, 1993; Evans *et al.*, 1992). The MOP, DOP and KOP receptors are homologous to each other. The MOP receptor has ~68% sequence homology to the KOP receptor and ~63% homology to the DOP receptor (Chuang *et al.*, 1995). There is substantial pharmacological evidence suggesting that additional receptor phenotypes may exist. However, to date no cDNA corresponding to further receptor subtypes have been identified. It has been hypothesised that the observed pharmacology could result from posttranslational modifications, alternative mRNA splicing (Pasternak, 2001) or homo- and hetero-oligomerisation (Jordan and Devi, 1999) of the identified receptor subtypes yielding unique pharmacological profiles.

Opioid receptors are activated by endogenously produced opioid peptides and by exogenously administered compounds. Endogenous opioid agonists include endorphin-1 and 2 that are selective agonists for the MOP receptor and dynorphin which acts as a selective agonist for the KOP receptor (Bradbury *et al.*, 1976; Cox *et al.*, 1976). The pro-enkephalin products leu- and met- enkephalin demonstrate the highest affinity for DOP receptors (Hughes *et al.*, 1975). The pro-opiomelanocortin product β -endorphin interacts with high affinity with MOP and DOP receptors.

Activation of opioid receptors can cause a variety of physiological effects including bradycardia, general sedation and depression of flexor reflexes (Jordan *et al.*, 2000). Opioid receptors are of great interest to pharmaceutical companies as they have been demonstrated to influence pain control. Activation of opioid receptors by ligand has been shown to inhibit neurotransmitter release from dorsal root ganglion projections in the dorsal horn of the spinal cord (MacDonald and Nelson, 1978; Mudge *et al.*, 1979; Yaksh, 1993).

The three subtypes of opioid receptor have been shown to form homo- and hetero-oligomers. Co-immunoprecipitation and BRET studies have demonstrated the existence of DOP receptor homo-oligomers (Cveijic *et al.*, 1997; McVey *et al.*, 2001). The KOP receptor has also been shown to form homo-oligomers and hetero-oligomer formation between KOP and DOP receptors has also been demonstrated (Jordan *et al.*, 1999). Interestingly, KOP and MOP receptors do not appear to form hetero-oligomers. Hetero-oligomer formation between MOP and DOP has been shown and displayed a unique pharmacological profile (George *et al.*, 2000; Gomes *et al.*, 2000). Whereas opioid receptors generally couple to G_i , hetero-oligomer formation resulted in receptor signalling that was insensitive to pertussis toxin treatment. This suggested unique G protein coupling to G proteins other than G_i/G_o (George *et al.*, 2000). A recent study performed by Gomes *et al.*, (2004) demonstrated that DOP receptor occupancy by an antagonist enhanced MOP receptor binding and signalling activity. The study also demonstrated that DOP receptor antagonists potentiate morphine-mediated analgesia.

Opioid and chemokine receptors have been shown to have overlapping expression patterns. Functionally active opioid receptors have been identified in immune cells also expressing

chemokine receptors and expression of both opioid and chemokine receptors have been demonstrated on glial cells in the brain (McCarthy *et al.*, 2001). Activation of opioid receptors has been demonstrated to modulate antibody and cellular immune responses (Taub *et al.*, 1991; Pellis *et al.*, 1986), inhibit natural killer cell activity (Weber *et al.*, 1989), modulate cytokine expression (Chao *et al.*, 1993; Peterson *et al.*, 1987; Belkowski *et al.*, 1995) and inhibit phagocytic activity (Szabo *et al.*, 1993; Rojavin *et al.*, 1993). The general immunosuppressive effects of opioid receptor activation may account for the increased incidence of infection observed in heroin addicts that was originally attributed to the use of non-sterile needles and impurities in the drug. Opioids also possess chemotactic activity and receptor activation can induce the chemotaxis of monocytes and neutrophils (Van Epps *et al.*, 1984; Grimm *et al.*, 1998). Opioid agonists also have the capacity to alter the expression of a large number of cytokines and cytokine receptors (Miyagi *et al.*, 2000). The MOP receptor agonist DAMGO ([D-ala², N-Me-Phe⁴, Gly-ol⁵] enkephalin) has been shown to produce a dose-dependent induction of the pro-inflammatory cytokines CCL5, CCL2 and CXCL10 (Wetzel *et al.*, 2000). Studies performed in cells infected with HIV-1 also demonstrate the expression of these pro-inflammatory chemokines following exposure to DAMGO. This effect is thought to assist infection of HIV-1 by mobilising uninfected cells to the sites of HIV-1 infection (Stecle *et al.*, 2003). DAMGO and morphine have also been found to increase expression of CXCR4 and CCR5, the HIV-1 co-receptors, in several cell types including astroglial cells and monocytes (Mahajan *et al.*, 2002).

Chemokines have been shown to participate in both normal physiological processes and pathological responses in the brain. The expression of several chemokine receptors, including CXCR2, has been shown within several regions of the brain including neurons in the hippocampus, regions of the cerebral cortex, amygdala, thalamus and basal ganglia

(Gabuzda *et al.*, 1998; Meucci *et al.*, 1998; Bajetto *et al.*, 1999). The production of IL-8 and GRO- α in the brain under normal physiological conditions has also been shown (Bajetto *et al.*, 2001; Robinson *et al.*, 1998). GRO- α also plays an important role in brain development as it synergises with platelet derived growth factor to promote the growth of immature oligodendrocytes (Robinson *et al.*, 1998).

Cross-desensitisation has been reported between several opioid and chemokine receptors. Both MOP and DOP receptor agonists have been found to induce the de-sensitisation of the chemokine receptors CCR1, CCR2, CXCR1 and CXCR2 (Grimm *et al.*, 1998). Additionally, treatment with the DOP receptor agonist met-enkephalin was found to induce cross-phosphorylation of both CXCR1 and CXCR2. Cross-desensitisation has also been reported for opioid receptors following exposure to chemokines. Exposure of several cell types to chemokines activating CCR2, CCR5, CCR7 and CXCR4 resulted in desensitisation of the chemotactic activity of MOP and DOP receptors (Szabo *et al.*, 2002). However, activation of CXCR1 and CXCR2 did not result in opioid receptor desensitisation. It has been suggested that a hierarchy exists in producing desensitisation that is inversely correlated with their susceptibility to desensitisation (Ali *et al.*, 1999). Results have shown that CXCR1 and CXCR2 are poor desensitisers but are relatively susceptible to desensitisation by other GPCRs (Ali *et al.*, 1999; Tomhave *et al.*, 1994). Exceptions have been noted in that CXCR1 and CXCR2 are capable of inducing cross-phosphorylation and cross-desensitisation of CCR1 in the human basophil cell line, RBL-2H3 (Richardson *et al.*, 2000). Interestingly CCR1 could cross-desensitise CXCR2 but not CXCR1, leading to the hypothesis that the duration of signalling induced by ligand activation of CCR1 was insufficient to cross-desensitise CXCR1. This theory is supported

by results demonstrating that phosphorylation deficient mutants of CCR1 that deliver a more sustained signal can desensitise CXCR1.

The observed cross-desensitisation occurring between the chemokine and opioid receptors may be indicative of an interaction occurring. Recently hetero-oligomerisation between CCR5 and MOP, DOP and KOP receptors has been demonstrated (Chen *et al.*, 2004; Suzuki *et al.*, 2002). Chen *et al.*, (2004) utilised a co-immunoprecipitation approach to demonstrate hetero-oligomers formed between CCR5 and MOP receptors. Suzuki *et al.*, (2002) demonstrated CCR5 hetero-oligomerisation with MOP, DOP and KOP receptors also using a co-immunoprecipitation approach.

It has been demonstrated that CXCR2 ligands can induce chemotaxis of PMNs containing opioids to the site of inflammation where they mediate anti-nociception (Brack *et al.*, 2004). Interestingly, it was also shown that almost all opioid-containing leukocytes expressed CXCR2. The aim in this chapter was to examine hetero-oligomerisation between CXCR2 and MOP, KOP and DOP receptors. This has been shown using co-immunoprecipitation and single cell FRET.

5.2 Co-immunoprecipitation of CXCR2 with MOP, KOP and DOP receptors

To investigate hetero-oligomerisation occurring between CXCR2 and MOP, MOP-YFP and Flag-CXCR2 were transiently expressed in HEK293T cells both individually and together. A HEK293T control was included as was a control composed of cells individually expressing the two receptors that had been mixed prior to

immunoprecipitation with an anti-GFP antibody that recognises YFP. Immunoprecipitated material was subjected to SDS-PAGE and detected by western blotting using an anti-Flag antibody. Figure 5.1 demonstrates the hetero-oligomer detected between CXCR2 and MOP. A band corresponding to the 38kDa CXCR2 receptor was detected in the lane corresponding to the sample in which the two receptors had been co-expressed. Bands corresponding to the predicted molecular weight of a dimer and higher order oligomers can also be observed, suggesting that a proportion of the CXCR2-MOP hetero-oligomer was not fully separated by the SDS-PAGE conditions used. No immunoreactivity was observed in the control sample in which cells expressing the two receptors individually were mixed prior to immunoprecipitation, suggesting that the hetero-oligomers observed are not an artefact of the protocol. No immunoreactivity was observed when the receptors were expressed individually. Appropriate receptor expression was confirmed by western blot analysis of cell lysates and detection of YFP fluorescence using a Victor² plate reader.

This protocol was repeated to detect hetero-oligomerisation between Flag-CXCR2 and KOP-YFP (Figure 5.2). Immunoreactivity at 38kDa corresponding to Flag-CXCR2 was observed in the lane corresponding to receptors co-expression suggesting hetero-oligomer formation. Interestingly, no immunoreactivity corresponding to higher order oligomers was detected suggesting that the CXCR2/KOP hetero-oligomer was fully separated by the SDS-PAGE conditions employed. As observed previously, no immunoreactivity was observed in the sample in which the individually expressed receptors had been mixed prior to immunoprecipitation, demonstrating that the hetero-oligomer observed is not simply an artefact of the conditions used in the experiment.

Hetero-oligomer formation between c-Myc-DOP and Flag-CXCR2 was investigated using this technique (Figure 5.3). Samples were immunoprecipitated with anti-Flag antibody and the immunoprecipitated material subjected to SDS-PAGE and western blotting analysis using an anti-c-Myc antibody. Immunoreactivity corresponding to the 60kDa c-Myc-DOP receptor was observed. Immunoreactivity was also observed at 30kDa and multiple bands at greater than 75kDa. No immunoreactivity was observed when the individually tagged receptors were expressed alone or mixed prior to immunoprecipitation. Western blots of cell lysates are shown to confirm correct expression.

5.3 Hetero-oligomerisation of CXCR2 with MOP, KOP and DOP detected using FRET in living cells.

Hetero-oligomerisation between CXCR2 and MOP, KOP and DOP was assessed using single cell FRET performed in live cells. As shown in Figure 5.4, FRET corresponding to CXCR2-CFP expressed alone was 0.014 ± 0.001 . Upon co-expression of CXCR2-CFP and MOP-YFP, a FRET signal of 0.33 ± 0.03 was observed. Similar levels of donor and acceptor fluorescence were observed as shown in Figure 5.4. Co-expression of CXCR2-CFP and KOP-YFP yielded an energy transfer of 0.4 ± 0.04 shown in Figure 5.5. However, it can be observed that the KOP-YFP receptor conjugate was not expressed at equivalent fluorescence levels to the CXCR2-CFP donor. As FRET is an acceptor dependent process this may mean that the energy transfer reported is lower than could be expected if both donor and acceptor were present at an equal ratio. Hetero-oligomerisation between CXCR2 and DOP was also investigated. CXCR2-CFP and DOP-YFP yielded a FRET signal of 0.41 ± 0.02 , shown in Figure 5.6. Similar levels of donor and acceptor fluorescent moieties were observed as shown in Figure 5.6.

5.4 Discussion

In this chapter, co-immunoprecipitation results indicated that CXCR2 can form hetero-oligomers with each of MOP, DOP and KOP receptors. A control in which cells expressing each epitope tagged receptor individually were mixed prior to immunoprecipitation was included and yielded no immunoreactivity. This demonstrates that the positive result observed for the interaction between CXCR2 and the opioid receptors was not an artefact of the immunoprecipitation protocol. Figure 5.2 represents the interaction observed between CXCR2 and the KOP receptor that was fully sensitive to the SDS-PAGE conditions used in this study, yielding a single band at the molecular mass corresponding to the CXCR2 monomer. Figures 5.1 and 5.3 demonstrate that the hetero-oligomers detected between CXCR2 and MOP and DOP receptors respectively were not fully separated by the SDS-PAGE conditions employed. Immunoreactivity corresponding to the predicted molecular mass of both the receptor monomer and higher order oligomers was observed, indicating that a proportion of the hetero-oligomers are resistant to separation by SDS-PAGE. SDS-resistant oligomers were also observed in chapter 4 representing CXCR1 and CXCR2 homo- and hetero-oligomers. The SDS-PAGE conditions used in this study may not have been sufficient to fully reduce all the hetero-oligomers present leading to the observation of apparent SDS-insensitive oligomers. In this study, fluorescent assessment of the KOP-YFP receptor has indicated that it is expressed at comparatively lower levels than DOP-YFP and MOP-YFP receptors in heterologous systems and therefore the lower levels of CXCR2/KOP hetero-oligomer formed may have been within a range to be fully reduced by the SDS-PAGE conditions. Additional immunoreactivity was observed for c-Myc-DOP that could not be explained by higher order oligomers. Immunoreactivity was observed at

30kDa and 75kDa that does not correspond to the predicted mass of the receptor. Suzuki *et al.*, (2002) also observed multiple immunoreactive bands representing DOP receptor when investigating expression in a human B cell line constitutively expressing opioid receptors. A similar pattern of immunoreactivity has also been observed corresponding to DOP receptor expressed by activated murine splenocytes (Miller *et al.*, 1998).

Single cell FRET experiments performed in this study further indicated that hetero-oligomerisation may occur between CXCR2 and the opioid receptors. The levels of FRET observed were much lower than that observed for the CXCR1/2 homo- and hetero-oligomer but greater than the energy transfer monitored between either CXCR1 /CXCR2 and the α_{1A} -adrenoceptor. This could be due to the conformation of the hetero-oligomer being such that there is a large distance between the donor and acceptor fluorescent moieties rather than the levels of hetero-oligomer formed being less than observed for the CXCR1/CXCR2 homo- and hetero-oligomers. To fully investigate the relative affinity of the CXCR2-opioid hetero-oligomers, saturation BRET experiments could be employed which would yield data indicating the relative propensity of the hetero-oligomers to form.

Hetero-oligomerisation between chemokine and opioid receptors has been shown previously. CCR5 has been shown to form hetero-oligomers with MOP, DOP and KOP receptors using co-immunoprecipitation techniques on monkey and human lymphocytes (Suzuki *et al.*, 2002). This study also employed chemical cross-linking to demonstrate that the hetero-oligomers exist on the cell surface as a complex with an intramolecular distance much less than 11.4Å. Agonist stimulation of either receptor within the complex had no effect on the immunoreactivity observed.

A recent study performed by Chen *et al.*, (2004) demonstrated CCR5 and MOP receptor hetero-oligomerisation using co-immunoprecipitation. This study also reported no effect of agonist on the level of immunoreactivity observed. Interestingly, this study yielded data suggesting that the activation of either CCR5 or MOP affected the G protein coupling of the other assessed by GTP γ S binding. This effect was attributed to the enhanced phosphorylation of CCR5 or MOP upon stimulation with agonist for the opposite receptor.

The relationship between pain and inflammatory conditions is well acknowledged. Upon the receipt of an injury, an inflammatory response is triggered that involves the migration of immune cells to the injury site. These immune cells can release a range of substances that include cytokines and chemokines. Both cytokines and chemokines can influence the sensation of pain by sensitisation of the dorsal horn neurons in the spinal chord (Sacerdote *et al.*, 2000). Chemokines can also influence pain perception in the brain. A study performed by Giovannelli *et al.*, (1998) demonstrated directly the participation of IL-8 and GRO- α in the regulation of neuronal transmission. In *in vitro* studies, exposure to these chemokines was found to enhance postsynaptic currents and reduce the magnitude of neurotransmitter release from Purkinje neurons. Hyperanalgesia has been demonstrated to occur during the inflammatory response (Watkins *et al.*, 1995; Junger and Sorkin, 2000). Recent human studies have confirmed results obtained from several animal experiments detailing that MOP receptors in the brain are crucial for the sensation of sustained peripheral pain (Zubieta *et al.*, 2001). In inflammation states, elevated levels of chemokines are detected in the brain causing altered neuronal function and reduced MOP receptor-mediated analgesia. This has led to the theory that the decreased analgesia may contribute to the pain in the periphery that is associated with a number of inflammatory diseases including rheumatoid arthritis (Szabo *et al.*, 2002). The presence of chemokine:

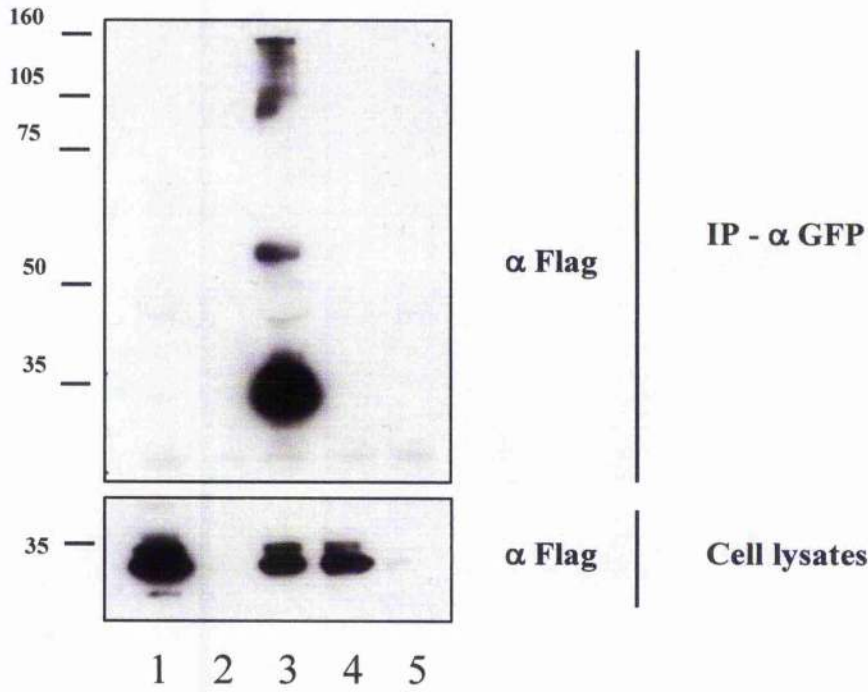
opioid hetero-oligomers suggests a new therapeutic route for the treatment of inflammatory conditions.

Figure 5.1 Co-immunoprecipitation of epitope-tagged forms of the human CXCR2 and MOP receptors

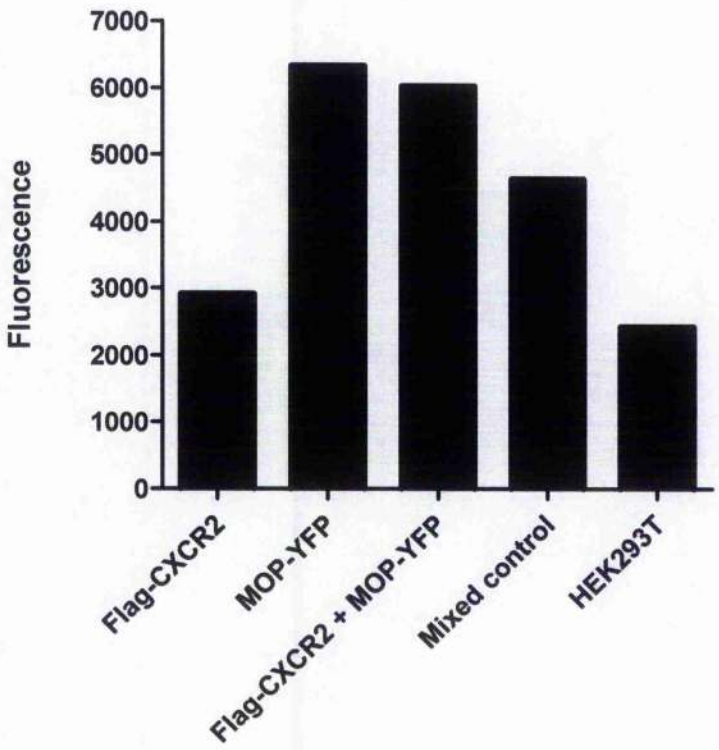
HEK293 cells were transfected to transiently express Flag-CXCR2 (lane 1) or MOP-YFP (lane 2). Flag-CXCR2 and MOP-YFP were coexpressed (lane 3). A non-transfected HEK293T (lane 5) and a mixed cell control (lane 4) were included. The mixed cell control represents cells individually expressing the constructs and mixed prior to immunoprecipitation. Cell lysates were immunoprecipitated with an anti-GFP antiserum that also recognises YFP, samples resolved by SDS-PAGE and then immunoblotted with anti-Flag antibody (upper panel). Western blot analysis of cell lysates using anti-Flag antibody was also performed in order to ensure correct protein expression (lower panel). Expression of MOP-YFP was detected by monitoring cell lysate fluorescence using a Victor² plate reader (b). One experiment representative of three experiments is shown.

Figure 5.1

Mr (x10⁻³)



(a)



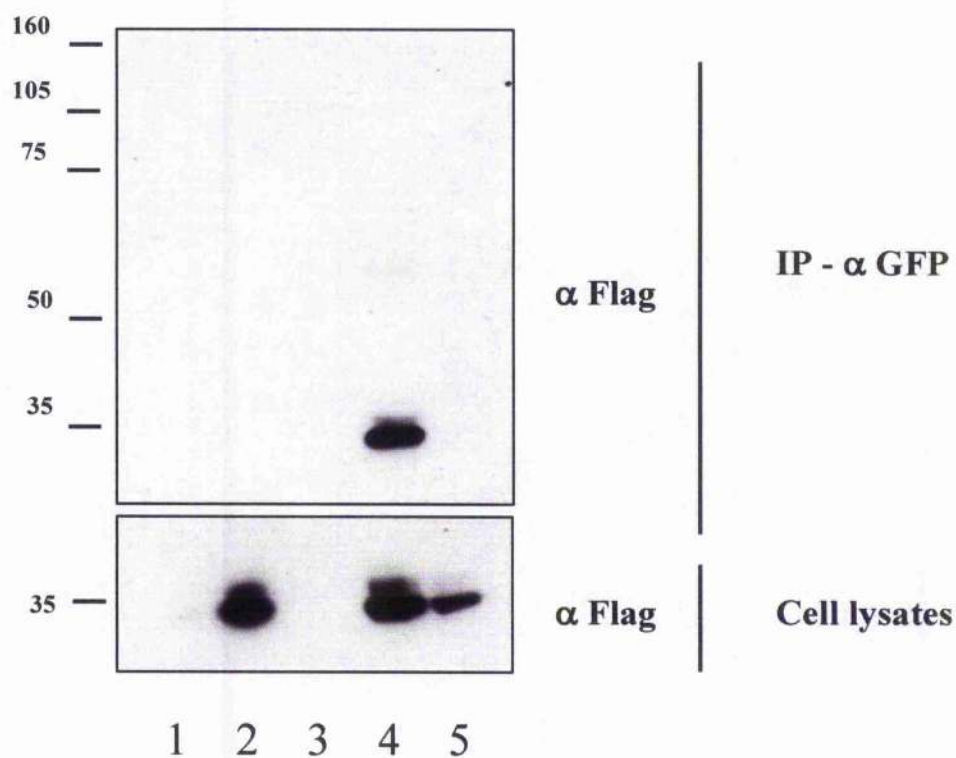
(b)

Figure 5.2 Co-immunoprecipitation of epitope-tagged forms of the human CXCR2 and KOP receptors

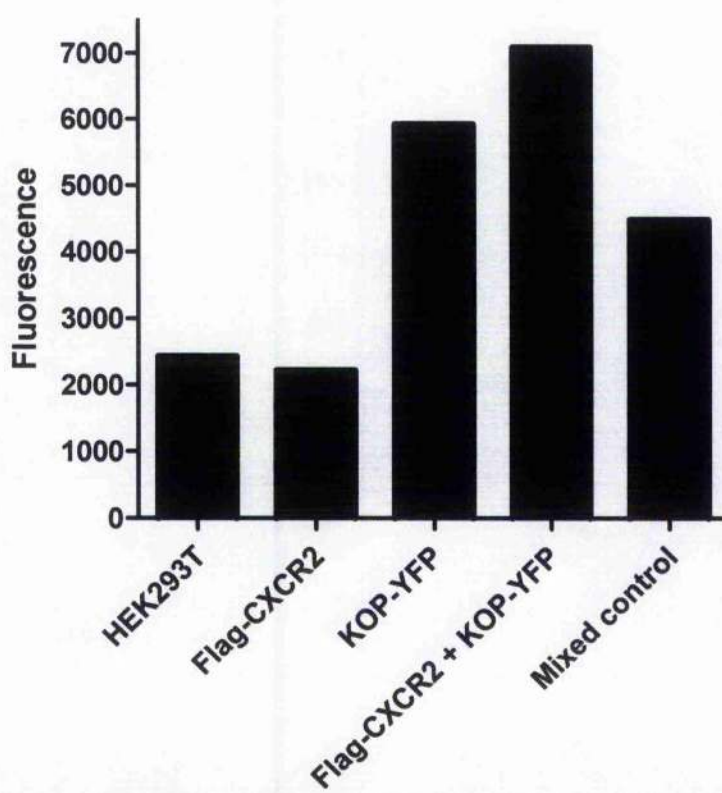
HEK293 cells were transfected to transiently express Flag-CXCR2 (lane 2) or KOP-YFP (lane 3). Flag-CXCR2 and KOP-YFP were coexpressed (lane 4). A non-transfected HEK293T (lane 1) and a mixed cell control (lane 5) were included. The mixed cell control represents cells individually expressing the constructs and mixed prior to immunoprecipitation. Cell lysates were immunoprecipitated with an anti-GFP antiserum that also recognises YFP, samples resolved by SDS-PAGE and then immunoblotted with anti-Flag antibody (upper panel). Western blot analysis of cell lysates using anti-Flag antibody was also performed in order to ensure correct protein expression (lower panel). Expression of KOP-YFP was detected by monitoring cell lysate fluorescence using a Victor² plate reader (b). One experiment representative of three experiments is shown.

Figure 5.2

Mr (x10⁻³)



(a)



(b)

Figure 5.3 Co-immunoprecipitation of epitope-tagged forms of the human CXCR2 and DOP receptors

HEK293 cells were transfected to transiently express Flag-CXCR2 (lane 2) or c-Myc-DOP (lane 3). Flag-CXCR2 and c-Myc-DOP were coexpressed (lane 4). A non-transfected HEK293T (lane 1) and a mixed cell control (lane 5) were included. The mixed cell control represents cells individually expressing the constructs and mixed prior to immunoprecipitation. Cell lysates were immunoprecipitated with anti-Flag antibody, samples resolved by SDS-PAGE and then immunoblotted with anti-c-Myc antibody (upper panel). Western blot analysis of cell lysates using anti-Flag and anti-c-Myc antibodies was also performed in order to ensure correct protein expression (lower panels). One experiment representative of three experiments is shown.

Figure 5.3

Mr (x10⁻³)

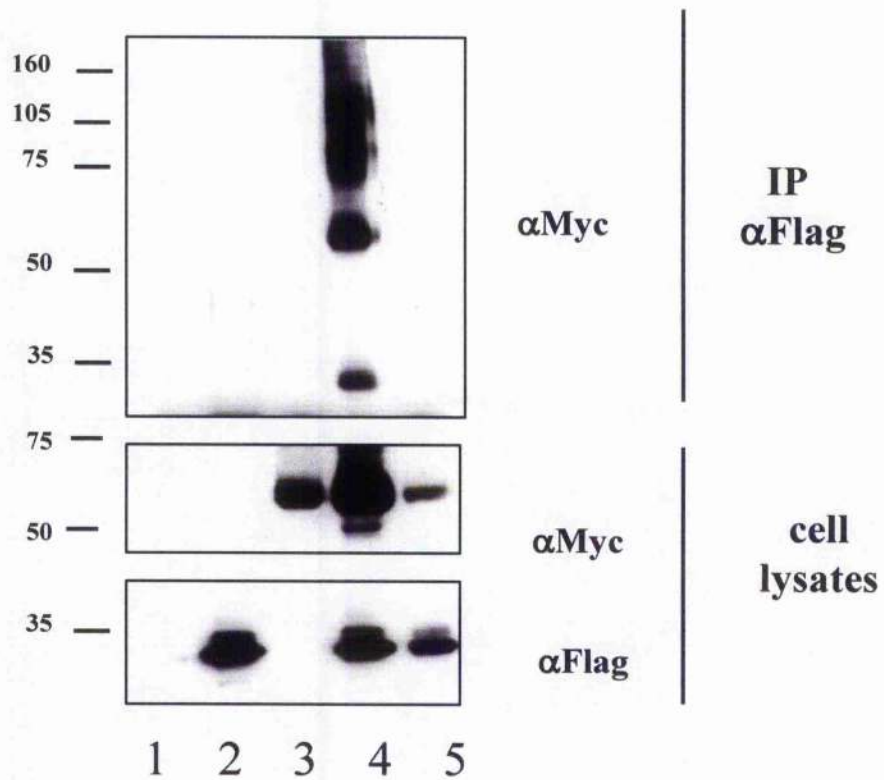


Figure 5.4 FRET imaging of constitutive CXCR2 and MOP hetero-oligomerisation in single cells

CXCR2-CFP and MOP-YFP were co-expressed (b) or MOP-YFP expressed individually (a) in HEK293T cells. Left hand panels represent CFP images, centre panels represent YFP images and right hand panels represent corrected FRET. FRET signals ($\text{FRET}_{\text{NORM}}$) were quantified as described in section 2.6.9 and are shown in (c). Data shown are mean \pm S.E.M. from three experiments.

Figure 5.4

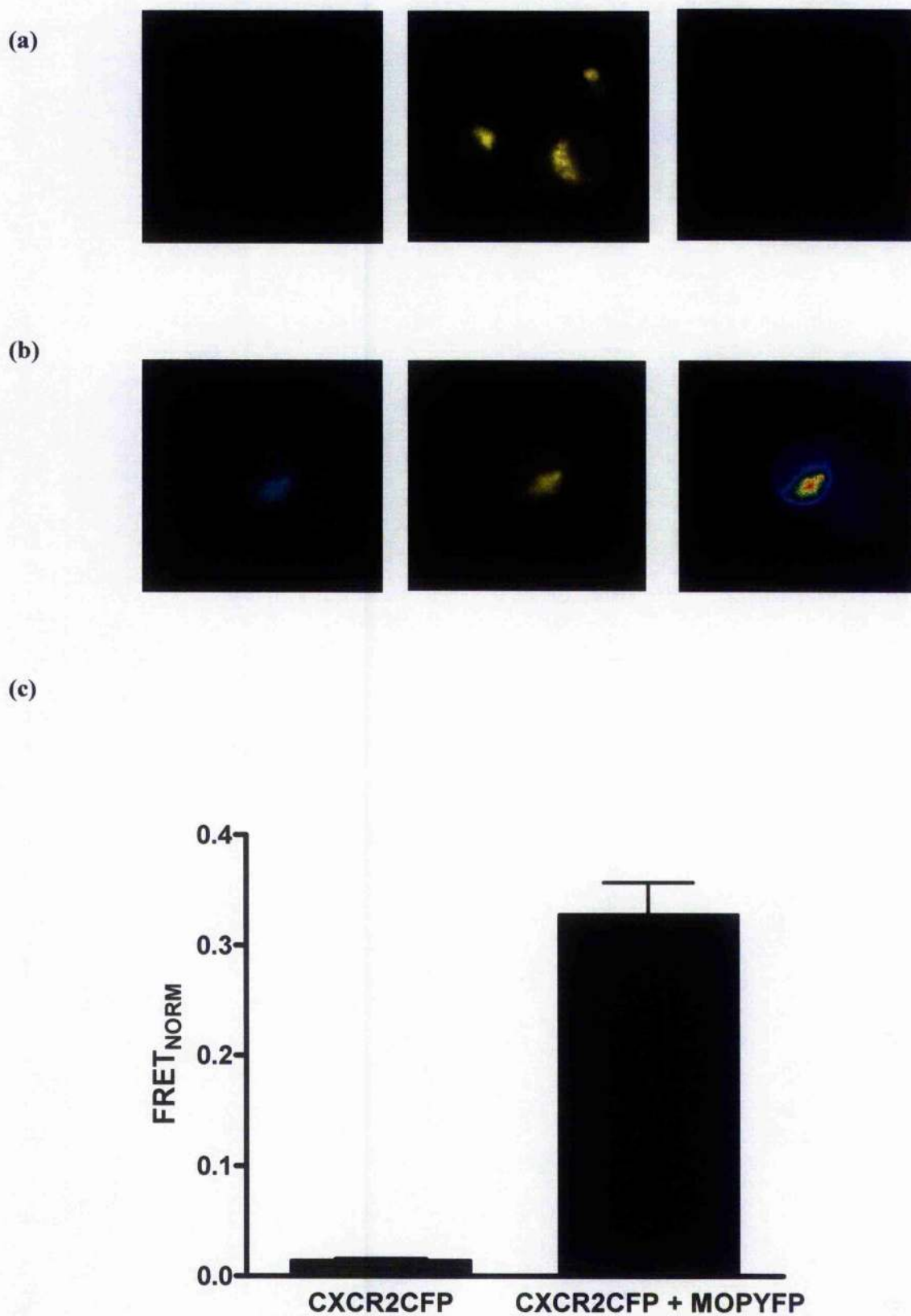


Figure 5.5 FRET imaging of constitutive CXCR2 and KOP hetero-oligomerisation in single cells

CXCR2-CFP and KOP-YFP were co-expressed (b) or KOP-YFP expressed individually (a) in HEK293T cells. Left hand panels represent CFP images, centre panels represent YFP images and right hand panels represent corrected FRET. FRET signals ($\text{FRET}_{\text{NORM}}$) were quantified as described in section 2.6.9 and are shown in (c). Data shown are mean \pm S.E.M. from three experiments.

Figure 5.5

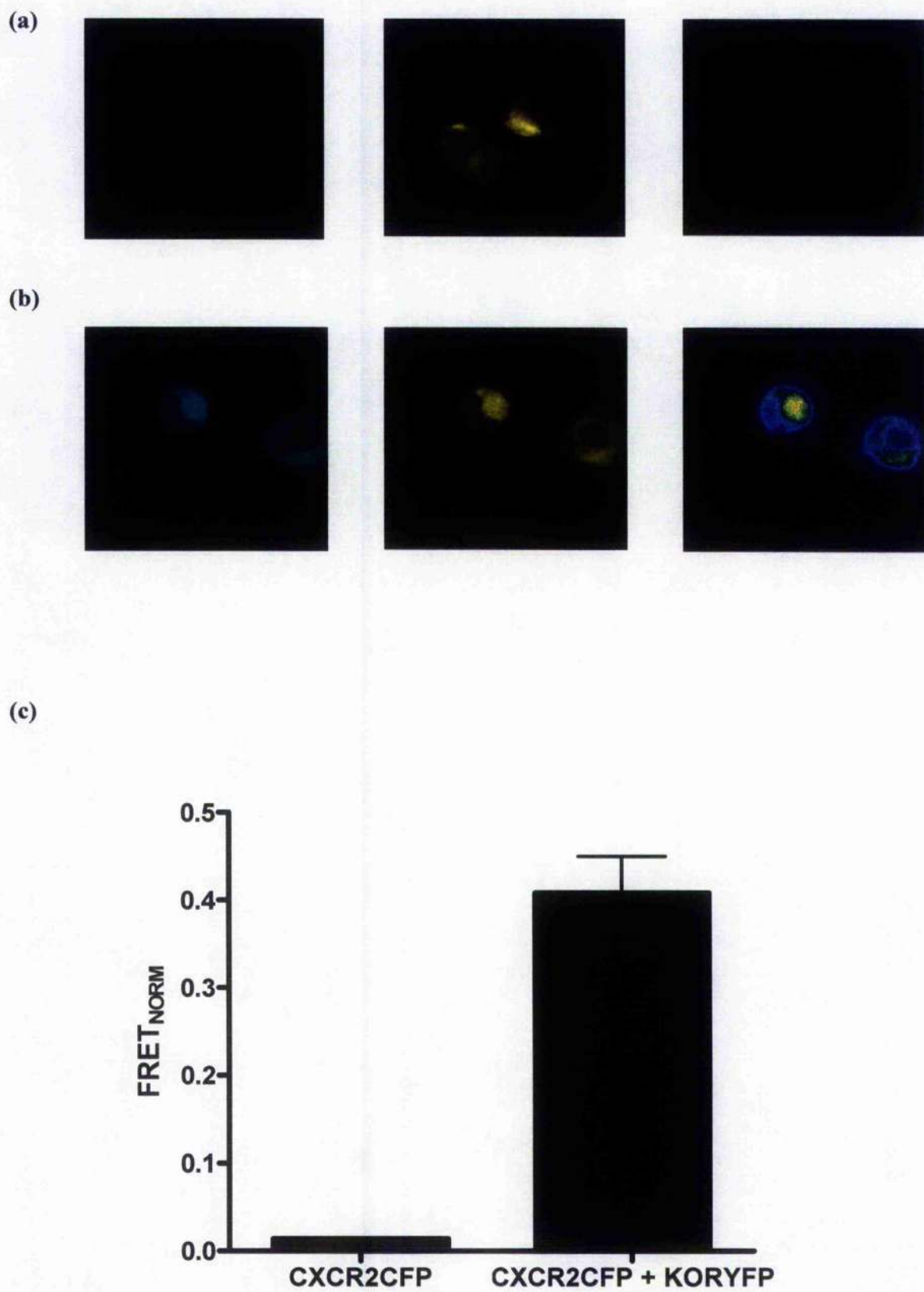
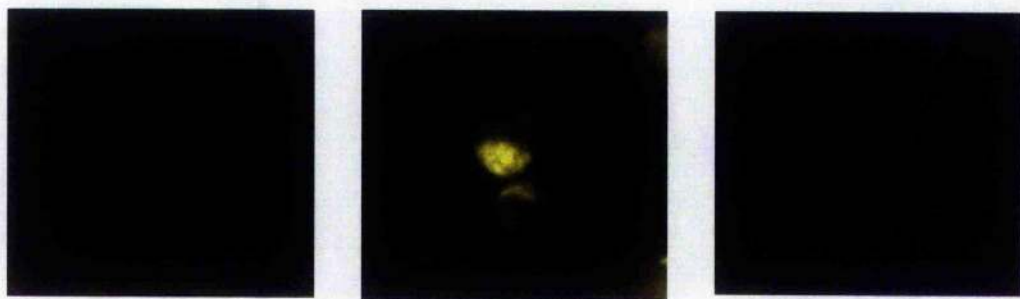


Figure 5.6 FRET imaging of constitutive CXCR2 and DOP hetero-oligomerisation in single cells

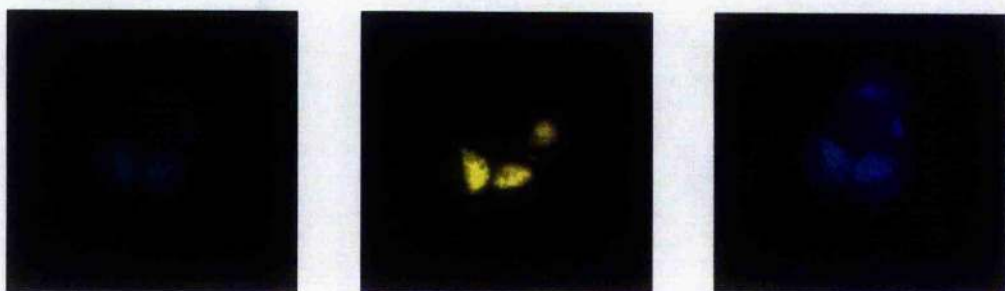
CXCR2-CFP and DOP-YFP were co-expressed (b) or DOP-YFP expressed individually (a) in HEK293T cells. Left hand panels represent CFP images, centre panels represent YFP images and right hand panels represent corrected FRET. FRET signals ($\text{FRET}_{\text{NORM}}$) were quantified as described in section 2.6.9 and are shown in (c). Data shown are mean \pm S.E.M. from three experiments.

Figure 5.6

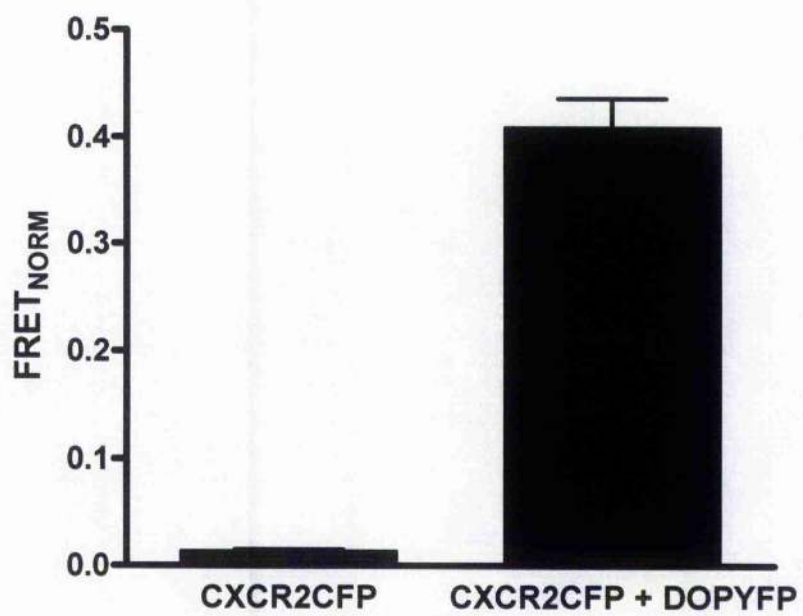
(a)



(b)



(c)



Chapter 6 Final Discussion

CXCR1 and CXCR2 have been implicated in several inflammation disease states including psoriasis and ulcerative colitis (Nickoloff *et al.*, 1991; Bizzarri *et al.*, 2003; Mahida *et al.*, 1992). Therefore, these receptors represent potential drug targets for pharmaceutical intervention in these conditions. Chemokine receptor oligomerisation has been demonstrated to be physiologically relevant. Oligomerisation of CCR5 with the naturally occurring CCR5 mutant *ccr5-32Δ* conferred a delay in progression of HIV-1 to AIDS (Benkirane *et al.*, 1997). A similar protective effect was observed in individuals possessing a mutant form of CCR2 termed CCR2V64I that was attributed to oligomerisation of this receptor with CCR5 or CXCR4 (Mellado *et al.*, 1999).

In this study, constitutive homo- and hetero-oligomerisation of CXCR1 and CXCR2 has been comprehensively demonstrated using several biochemical and biophysical techniques including co-immunoprecipitation, single-cell FRET, time resolved FRET and BRET. Each technique indicated that both CXCR1 and CXCR2 homo-and hetero-oligomers are constitutively formed. The selectivity of oligomerisation was investigated in this study. Saturation BRET experiments indicated that CXCR1/CXCR2 homo- and hetero-oligomers form with equal propensity as indicated by similar $[A]/[D]$ ratios observed at half maximal BRET signals. Saturation BRET experiments have been used extensively in an attempt to quantify the likelihood of a GPCR pair to form oligomers (Canals *et al.*, 2004; Canals *et al.*, 2003; Mercier *et al.*, 2002; Breit *et al.*, 2004; Ayoub *et al.*, 2004; Terrillon *et al.*, 2003). Ramsay *et al.*, (2004) utilised this technique to determine the specificity of α_{1A} -adrenoceptor homo-oligomerisation. The authors demonstrated that interactions between the α_{1A} -adrenoceptor and the DOP receptor were some 75 times lower affinity than

observed for the α_{1A} -adrenoceptor homo-oligomer. Many studies investigating GPCR oligomerisation have selected a GPCR with low sequence similarity to the receptor of interest to act as a negative control. The α_{1A} -adrenoceptor was selected as a control in this study. A recent phylogenetic analysis identified both CXCR1/CXCR2 and the α_{1A} -adrenoceptor as members of the rhodopsin family (Fredriksson et al., 2003). However, further analysis demonstrated that the rhodopsin-like receptors could be further subdivided into four groups of which there were 13 distinct branches. The α_{1A} -adrenoceptor was found to be a member of the α -group of rhodopsin receptors within an amine receptor cluster. CXCR1 and CXCR2 were found to be members of the γ -group of rhodopsin receptors within the chemokine receptor cluster. This suggests that although both the chemokine and the α_{1A} -adrenoceptors share a common evolutionary origin, they do not share significant sequence similarity. CXCR1/CXCR2 and the α_{1A} -adrenoceptor have not been shown to share an overlapping expression pattern therefore this receptor was selected to act as a negative control in this study. Oligomerisation between CXCR1/CXCR2 and the α_{1A} -adrenoceptor was investigated using Tr-FRET, single cell FRET and BRET. Some energy transfer was detected using Tr-FRET and single cell FRET, however, BRET saturation experiments suggested that this interaction was non-specific as a linear relationship existed between signal and acceptor/donor expression ratio. The ER retention assay also demonstrated that CXCR1 conjugated to an ER retention sequence did not prevent export of the α_{1A} -adrenoceptor from the ER to the cell surface and was interpreted as a lack of interaction between the two receptors. A further approach to investigate the selectivity of oligomerisation would have been to restrict receptor expression to levels comparable with that observed physiologically. Issafras *et al.*, (2002) utilised this approach in BRET studies investigating CCR5 and CXCR4 oligomerisation. At physiologically relevant expression levels, CCR5 and CXCR4 homo-oligomerisation could be observed however no hetero-

oligomerisation was observed. In this study receptor expression levels were not quantified, however, several studies have demonstrated the independence of receptor density and BRET at relatively low levels of receptor expression (Mercier *et al.*, 2002; Issafras *et al.*, 2002; Babcock *et al.*, 2003).

Oligomerisation monitored by Tr-FRET indicated that CXCR1 and CXCR2 homo- and hetero-oligomers exist at the cell surface. However, CXCR1 and CXCR2 hetero-oligomers were found not to undergo internalisation in response to GRO- α . This was surprising as saturation BRET experiments suggested that CXCR1/CXCR2 homo- and hetero-oligomers had an equal propensity to form. Similar experiments were performed in which one receptor within the hetero-oligomer complex was stably expressed and the other transiently expressed. The cells were stimulated with GRO- α 24 hours following transient transfection, however no co-internalisation was observed (data not shown). Subsequent experiments performed within the Milligan group have suggested that the 24-hour time frame following transfection and stimulation of receptors with drug may have been insufficient to permit the co-synthesis of both the stably and transiently expressed receptors. It would be interesting to create a stable cell line co-expressing both CXCR1 and CXCR2 and investigate co-internalisation. The Flp-In T-REX 293 system (Invitrogen) has been used successfully to create double stable cell lines within the Milligan group. This system permits the inclusion of one receptor that is constitutively expressed and one receptor to be expressed within a doxycycline inducible region. A 48-hour time period should be observed following doxycycline exposure to permit co-synthesis of the receptors. Also by using increasing concentrations of doxycycline, the expression level of the inducible receptor may be increased. This would allow the expression of one receptor within the hetero-oligomer to be expressed at higher levels than the other, potentially

favouring hetero-oligomer formation as opposed to homo-oligomerisation. This method may provide more information concerning the internalisation properties of the CXCR1 and CXCR2 hetero-oligomers.

An ER-trapping assay developed in this study revealed that CXCR1/CXCR2 homo- and hetero-oligomeric interactions began during protein synthesis and maturation. This assay could be adapted to investigate oligomerisation of other GPCRs. In this study the amount of cell surface receptor was quantified by incubation of whole cells in suspension with antibody conjugated to a fluorescent moiety. This assay could be adapted into an ELISA format performed on whole cells which would remove any antibody labelling of broken cells that would inevitably occur while performing the assay on cells in suspension. An ELISA format would also allow cell number to be regulated as a controlled number of cells would be sub-cultured into each well of a 96-well plate, permitting direct comparison of cell surface fluorescence.

Tr-FRET and BRET data indicated that oligomerisation was unaffected by the presence of agonist. However, this was assessed by comparing maximal energy transfer observed in the absence and presence of ligand. Any change in energy transfer observed could represent ligand binding inducing a conformational change within the oligomer resulting in the orientation of the donor and acceptor moieties to be more conducive to energy transfer. It would have been beneficial to examine any effect ligand had on the BRET₅₀ values generated in saturation BRET experiments, indicating change in affinity of the donor and acceptor for each other. The single cell FRET technique utilised in this study would have been very useful in identifying any effect ligand binding had on oligomerisation. BRET has the disadvantage of utilising the cell permeable substrate DeepBlueC that reports on

energy transfer occurring throughout the cell therefore any small alterations in energy transfer caused by ligand binding may be masked by energy transfer occurring between receptors within the cell that have not been exposed to ligand. Single cell FRET permits the measurement of energy transfer occurring only at the cell membrane exposed to ligand and would be highly useful in investigating this area of oligomerisation. Unfortunately, this technique requires a large amount of ligand that was not economically viable in this study.

Due to time constraints the functional properties of CXCR1/CXCR2 hetero-oligomerisation could not be investigated. Mellado *et al.*, (2001) outlined a hypothesis regarding chemokine receptor oligomerisation. This theory detailed that hetero-oligomerisation formation conferred unique pharmacology to the receptors involved such as G protein switching from $G\alpha_i$ to $G\alpha_{q/11}$, distinct PI3-K activation kinetics and preferential activation of cell adhesion. Hetero-oligomers were also shown to be resistant to internalisation and desensitisation to a second stimulus. This theory was based on chemokine receptor oligomerisation being ligand induced, however, several reports have now demonstrated constitutive chemokine receptor oligomerisation. Initial experiments investigating the influence of CXCR1/CXCR2 hetero-oligomer formation on CXCR2 ligand binding have been performed. These experiments have shown that the efficacy of GRO- α to inhibit forskolin-stimulated cAMP release in cells co-expressing CXCR1 and CXCR2 is no different than that observed for cells singly expressing CXCR1 and CXCR2 and mixed prior to the experiment. This suggests that CXCR2 ligands bind to CXCR1/CXCR2 hetero-oligomers and demonstrate no unique pharmacology. However, it would be beneficial to fully investigate and characterise the pharmacology conferred by the hetero-oligomers.

Hetero-oligomerisation between CXCR2 and opioid receptor subtypes was shown using co-immunoprecipitation and single cell FRET. Oligomerisation between these receptors is of great interest as physiologically both CXCR1 and CXCR2 are involved in mediating inflammation that is associated with pain. Due to time constraints it was not possible to investigate the pharmacology of these hetero-oligomers. This would be of great interest as previous reports have suggested that hetero-oligomerisation between opioid receptor subtypes results in G protein switching to G proteins other than $G\alpha_i/G\alpha_o$. It would be useful to investigate if any unique pharmacology was observed as a result of chemokine/opioid receptor interaction.

Several variations of RET were utilised in this study including BRET, FRET and Tr-FRET. Both the variations of FRET and TR-FRET used in this study would not be amenable to high-throughput screening commonly used by major pharmaceutical companies due to multiple washing steps and expensive equipment required. BRET has been adapted into a high-throughput system to allow investigation into the activation state of the insulin receptor. The action of insulin is mediated by a tyrosine kinase receptor that upon ligand binding undergoes a conformational change that brings the two β subunits of the receptor into close proximity. This change in conformation permits the trans-phosphorylation of one receptor β subunit by the other subunit (Combettes-Souverain and Issad, 1998). Phosphorylation stimulates the tyrosine kinase activity of the receptor. The BRET assay involved the generation of receptor chimeras where one receptor β subunit was fused to the energy donor Rluc and the other conjugated to the energy acceptor YFP. Any conformational change induced in the insulin receptor results in an increase in energy transfer (Boute *et al.*, 2001). High throughput screening in this manner permits the rapid screening of compounds thought to possess insulin-like activity (Boute *et al.*, 2002). In this

study ligand incubation had no effect on the BRET signal corresponding to CXCR1 and CXCR2 homo- and hetero-oligomers and would therefore not be a useful technique for pharmaceutical companies interested in activating the receptors within homo- or hetero-oligomeric complexes. However, if further functional studies were to indicate a therapeutic value in homo- or hetero-oligomer dissociation, this assay would provide a useful platform for the screening of multiple compounds.

A recent study performed by Hlavackova *et al.*, (2005) investigated the requirement for both heptahelical domains to be switched on upon activation of a dimeric class C GPCR, the mGluR₁ receptor. This study utilised the quality control system of the heterodimeric GABA_B receptor in which the GABAbR1 subunit contains a retention sequence within its C terminus, preventing the cell surface expression of this subunit. Upon coexpression with GABAbR2, the retention sequence is masked and the heterodimer is trafficked to the cell surface (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998; Keiner *et al.*, 1999, Sullivan *et al.*, 2000). The mGluR₁ receptor was modified by introducing the C terminal tails of GABAbR1 and GABAbR2. A mutation was made to intracellular loop 3 of the mGluR₁ receptor conjugated to the C terminal tail of GABAbR2 that had been shown to suppress the ability of the receptor to activate phospholipase C and adenylyl cyclase. This permitted the determination of mGluR₁ hetero-oligomer function as only hetero-oligomers would be trafficked to the cell surface with homo-oligomers being either retained intracellularly or non-functional. This system could potentially be adapted to investigate CXCR1 and CXCR2 hetero-oligomer function. In this study CXCR1 and CXCR2 hetero-oligomers were found to exist constitutively at the cell membrane. CXCR1 could be modified by the addition of the C terminal tail of GABAbR1 containing the retention sequence followed by the C-terminal tail region of the GABAbR2 receptor containing the

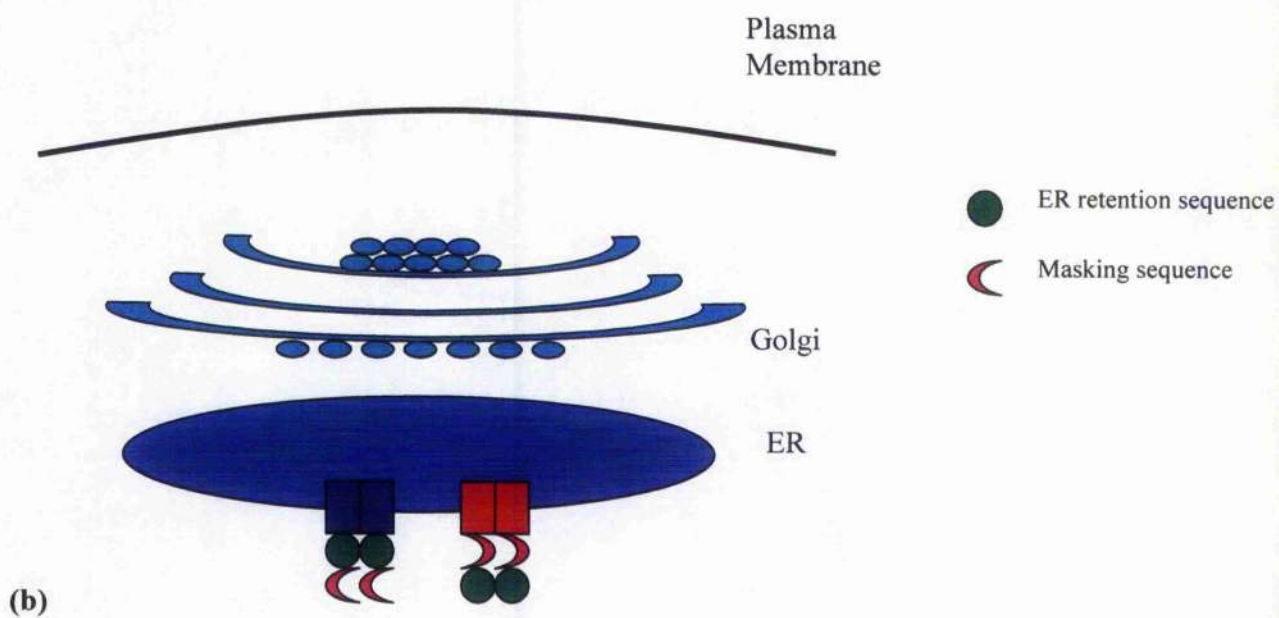
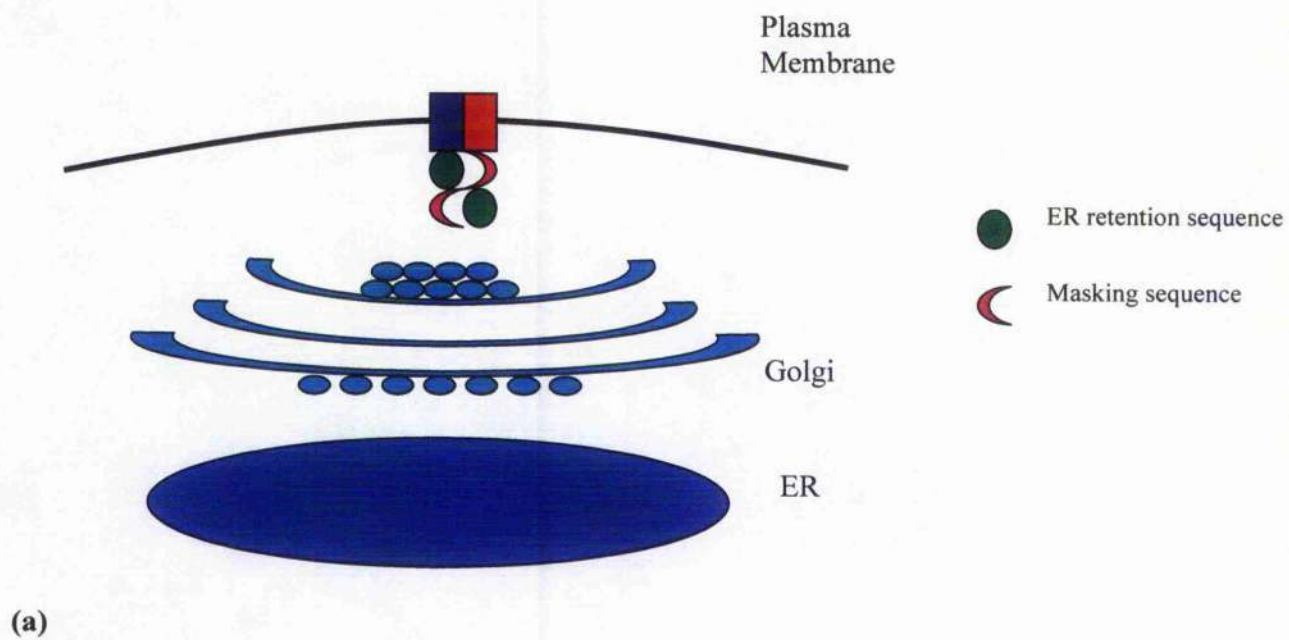
masking region. CXCR2 could be similarly modified but with the addition of the C terminal tail of GABABR2 containing the masking region proximal to the receptor followed by the C terminal tail region of GABABR1. This is illustrated in Figure 6.1. As the two receptors share high sequence homology and are similar in size, it could be predicted that the receptors would be synthesised in a manner allowing the combination of retention/masking sequences to act as a 'lock and key' mechanism allowing export from the ER. This would only permit the export of hetero-oligomers to the cell surface while homo-oligomers would be retained as the retention sequences present would not be masked. This would allow CXCR1/CXCR2 hetero-oligomers only to be studied and could potentially provide useful data regarding the physiological function of the hetero-oligomers.

As discussed previously, Hernanz-Falcon *et al.* (2004) demonstrated two amino acids present in TM I and IV of CCR5 (I52V and V150A) that were crucial for receptor oligomerisation and function. The study extended this analysis to identify two residues present in CCR2 (V64A and V164A) also crucial for receptor oligomerisation and function. Analysis of CXCR1 and CXCR2 has identified three analogous residues present within each receptor to which mutations could potentially abrogate oligomerisation. These have been identified as V63A, I64V and L158I in CXCR1 and V63A, I64V and L167I in CXCR2. Due to time constraints this could not be pursued however this would be very informative in determining the residues important in CXCR1 and CXCR2 oligomerisation and could potentially highlight a therapeutic target for oligomers. Hernanz-Falcon *et al.* (2004) also demonstrated that synthetic peptides generated to the residues in TM I and IV blocked CCR5 agonist induced signalling both *in vitro* and *in vivo*, demonstrating the physiological relevance of these residues.

In conclusion, the identification of CXCR1 and CXCR2 homo- and hetero-oligomers introduces further complexity to the chemokine signalling network.

Figure 6.1 Potential method of investigating hetero-oligomer pharmacology

A combination of ER retention/masking sequences derived from the GABA_B receptors has been suggested to permit characterisation of cell surface hetero-oligomer function. Receptors would be modified by the introduction of either the C-terminal region of GABA_BR1 containing the retention sequence or GABA_BR2 containing the masking sequence. Hetero-oligomers would possess the correct orientation of retention/masking sequences fused to the C terminal of the receptors to permit export from the ER to the cell surface (a) while homo-oligomers would be retained in the ER as the retention sequence would not be masked (b).



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